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CONTENTS

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PAGE

STEWARD, J. S. Anthelmintic studies: I. A controlled critical entero-nemacidal test 231

STEWARD, J. S. Anthelmintic studies: II. A double entero-nemacidal anthelmintic test covering a wide range of activities. (With 1 Figure in the Text) . . 242

STEWARD, J. S. Anthelmintic studies: III. A taeniacidal testing technique. . . 255

STEWARD, J. S. Anthelmintic studies: IV. The loss of efficiency by division of the dose 266

ZUMPT, F. and TILL, W. The mange-causing mites of the genus *Psorergates* (Acarina: (Myobiidae) with description of a new species from a South African monkey. (With 7 Figures in the Text) 269

RADFORD, CHARLES D. Some new and little-known mites of the genus *Myocoptes* Claparède (Acarina: Listrophoridae). (With 16 Figures in the Text) . . 275

MELLANBY, HELEN. The identification and estimation of acetylcholine in three parasitic nematodes (*Ascaris lumbricoides*, *Litomosoides carinii*, and the microfilariae of *Dirofilaria repens*). (With 6 Figures in the Text) 287

REES, GWENDOLEN. The adult and Diplostomulum stage (*Diplostomulum phoxini* (Faust)) of *Diplostomum pelmatoides* Dubois and an experimental demonstration of part of the life cycle. (With Plate V and 10 Figures in the Text) . 295

FRANKLAND, HELGA M. T. The life history and bionomics of *Diclidophora denticulata* (Trematoda: Monogenea). (With 21 Figures in the Text) 313

EVANS, G. OWEN. A review of the Laelaptid paraphages of the Myriapoda with descriptions of three new species (Acarina: Laelaptidae). (With 30 Figures in the Text) 352

WEBBER, WINFRITH A. F. *Dirofilaria aethiops* Webber, 1955, a filarial parasite of monkeys: I. The morphology of the adult worms and microfilariae. (With 12 Figures in the Text) 369

HAWKING, FRANK and WEBBER, WINFRITH A. F. *Dirofilaria aethiops* Webber, 1955, a filarial parasite of monkeys: II. Maintenance in the laboratory. (With 1 Figure in the Text) 378

WEBBER, WINFRITH A. F. *Dirofilaria aethiops* Webber, 1955, a filarial parasite of monkeys: III. The larval development in mosquitoes. (With 16 Figures in the Text) 388

WEBBER, WINFRITH A. F. and HAWKING, FRANK. The filarial worms *Dipetalonema digitatum* and *D. gracile* in monkeys. (With 7 Figures in the Text) . . . 401

COOK, EDWIN F. and BEER, JAMES R. The louse populations of some cricetid rodents. (With 4 Figures in the Text) 409

RAY, H. N. and DASGUPTA, B. Observations on the structure and the nature of '*NR* bodies' found in the testes of bed-bug. (With Plate VI) 421

SARWAR, M. M. On a new lungworm, *Pneumostrongylus ovis* Sarwar, 1953, and some remarks on *Varestrongylus* species from sheep and goats in Pakistan. (With 3 Figures in the Text) 426

INGLIS, WILLIAM G. On the family Parasubuluridae van den Berghe & Vuylsteke, 1938, and the subfamily Numidicinae López-Neyra, 1945 (Nematoda). (With 9 Figures in the Text) 431

GOODCHILD, A. J. P. The bacteria associated with *Triatoma infestans* and some other species of Reduviidae 441

MEYER, H. and MENDONÇA, I. DE ANDRADE. Electron microscopic observations of Toxoplasma 'Nicolle et Manceaux' grown in tissue cultures (first note). (With Plate VII) 449

MCCONNACHIE, ELSPETH W. Studies on *Entamoeba invadens* Rodhain, 1934, *in vitro*, and its relationship to some other species of *Entamoeba*. (With 13 Figures in the Text) 452

SMITH, KENNETH M. Intranuclear changes in the polyhedrosis of *Tipula paludosa* (Meig.). (With Plates VIII–XI) 482

Index of Authors 488

Index of Subjects 491

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ANTHELMINTIC STUDIES: I. A CONTROLLED CRITICAL ENTERO-NEMACIDAL TEST

BY J. S. STEWARD, F.R.C.V.S., D.V.S.M.
Nottingham

CONTENTS

	PAGE
I. Introduction	231
(*a*) Existing techniques	231
(*b*) The choice of test infection	233
II. The life-history of the test organism (*Heterakis spumosa*)	233
III. A comparison of the types of anthelmintic test	234
IV. Technique	235
(*a*) The development of worms in relation to infective dose	236
(*b*) The excretion of worms in untreated rats	236
V. The interpretation of anthelmintic activity	237
VI. The responses of the test organism (*Heterakis spumosa*) to known anthelmintics	239
VII. Discussion	240
Summary	241
References	241

I. INTRODUCTION

(*a*) *Existing techniques*

Most anthelmintics have been discovered either through the empirical application of selected compounds to man or domestic animals, or by the use of critical tests in dogs (e.g. carbon tetrachloride, Hall, 1921; tetrachlorethylene, Hall & Shillinger, 1925).

Some anthelmintics with a destructive action on or via the helminth cuticle were detected by *in vitro* testing with *Ascaris* (e.g. hexyl resorcinol, Lamson, Brown, Ward & Robbins, 1930; Lamson, Caldwell, Brown & Ward, 1930 and Lamson & Brown, 1936).

In several cases the insecticidal properties of particular compounds have led to their use as anthelmintics, e.g. phenothiazine (Harwood, Jesstad & Swanson, 1938), sodium fluoride (Habermann, Enzie & Foster, 1945) and nicotine.

The *in vitro* tests using intact *Ascaris* (Lamson & Brown, 1936), or tied off neuro-muscular preparations of *Ascaris* (Baldwin, 1943) may be used for the comparison of closely related chemical compounds possessing local destructive action or the ability to penetrate the cuticle. The later developed technique of Baldwin & Moyle (1947) with 'exposed' muscle preparations of *Ascaris* has been used to study the effect of compounds of physiological and pharmacological interest but does not appear to have been used as an anthelmintic sorting test.

The usual disadvantages of *in vitro* tests for chemotherapy are even greater when this method is used for anthelmintics, as the drugs have to travel variable distances along the gastro-intestinal tract before reaching the parasites which may be embedded in the mucous membrane of the gut or covered with mucus or other secretions. The particular disadvantage of the *in vitro* techniques using *Ascaris* as

the test organism is that because of the great specificity of response of worms to anthelmintics the results obtained with *Ascaris* cannot reasonably be translated to *Enterobius*, or other worms not closely related to the test worm or occurring in the same part of the intestine.

In vitro testing with *Ascaris* is of greatest value in the comparison or assay of a series of chemical compounds of similar constitution and might be used to select the most active compounds for trial in experimental animals and, later, man.

The main disadvantage of the *in vitro* testing of speculative compounds for anthelmintic activity is that one cannot be certain that either positive or negative results are genuine. Thus, for example, many *in vitro* positive results cannot be confirmed in the living host and phenothiazine and gentian violet are both inactive *in vitro*. In consequence, all compounds giving positive results and the more important ones giving negative results should be tested against appropriate infections in animals.

Another *in vitro* test organism is the vinegar eel worm, *Anguillula aceti*, which has been used by Vanni (1947).

Hall's critical anthelmintic testing was performed largely on dogs with natural infections (*Ascaris*, hookworm, *Trichuris* and tapeworm)—the numbers of worms excreted after treatment were compared with those surviving in the animal when it was slaughtered.

The trichostrongylid worm, *Nippostrongylus muris*, has been used in rats for detecting anthelmintic activity by Rogers (1944) and Whitlock (1943). The latter assayed the activity of a series of simple saturated hydrocarbons against the infection, and confirmed his findings by tests against heavy natural infections with trichostrongylids in sheep.

The technique of Whitlock was the usual type of chemotherapeutic procedure in which the infections of treated and untreated groups were compared and activity based on the difference on post-mortem examination of the infected rats. Rogers, however, used a perfusion technique of the isolated worm-infected gut, but concluded that 'for the detection of new anthelmintics the method has limitations, drugs active in perfusion are not necessarily active in the live animal, though drugs inactive in the perfused gut are probably inactive *in vivo*'.

An alternative method used by Rogers consisted of performing worm egg counts on samples of faeces on 3 days before and 2 days after treatment. The pre-treatment egg counts were used for predicting the expected number of worms and the parasite survival rate in the treated rats was computed from the pre-treatment egg count of the treated and the egg/worm ratio found in the untreated controls.

The reaction of *Nippostrongylus* to anthelmintics is similar to a number of the Trichostrongyles in ruminants, *Ascaris* and hookworms. It shows a variable response to phenothiazine and could not safely be relied upon to detect anthelmintics of the phenothiazine type for animals, or such as would be expected to act on threadworm (*Enterobius*) in man. For this purpose one would require a worm regularly susceptible to phenothiazine.

Deschiens & Larmy (1945) have used mixed natural infections of *Aspiculuris tetraptera*, *Syphacia obvelata* and other worms, and have demonstrated an effect

with diphenylamine derivatives on dosing for 8 consecutive days, and on *Passalurus ambiguus* in rabbits for 5 days.

Erhardt (1941) tested for the anthelmintic properties of compounds by using cats with multiple infections with *Opisthorchis* (trematodes in the liver and pancreas), *Strongyloides*, *Ancylostoma caninum* (hookworm), *Trichuris serrata* (whipworm), *Toxocara cati* (*Ascaris*), *Taenia taeniformis* and *Dipylidium caninum* (intestinal cestodes).

Erhardt & Gieser (1941) also used *Passalurus* infections in rabbits to detect compounds with oxyuricidal properties. On account of the difficulty of diagnosing this infection by ordinary methods they performed laparotomy to inspect the bowel and determine the presence and degree of the infection. The rabbits found to be infected are treated a few days after the operation.

(*b*) *The choice of test infection*

It was considered that an anthelmintic to be successful should act in one dose and that routine sorting tests should be based on a single dosage. It was also felt that the rat would be the most suitable experimental animal and that if a suitable worm responding to phenothiazine could be found in rats then that infection could be combined with *Nippostrongylus* to cover a much wider range of helminth reactions to anthelmintics than a single infection.

It seemed that *Heterakis* (*Ganguleterakis*) *spumosa* (Schneider, 1866) of the wild rat might be the infection required, and its presence had been recorded in England. Its life cycle had been studied by Winfield (1933) who showed that it responded (to some extent) to carbon tetrachloride. This worm does not appear to have received any further attention until the present investigation. Its close relationship to the caecal worm (*Heterakis gallinae*) of chickens indicated that it should respond to phenothiazine.

Wild rats were obtained through the kind co-operation of the Rodent Officer of the Cheshire County Council and after a series of post-mortem examinations had been made the worms were found in the large intestine of rats from Bollington, Cheshire. Cultures were made from these and the infection was established in laboratory (white) rats. When tested the infection was found to respond to phenothiazine and the worms excreted in the faeces were large enough to be seen. It was therefore possible to use this infection for critical testing. An additional advantage is that this worm does not occur naturally in laboratory white rats—unlike *Syphacia obvelata* which also is so small as to make the accurate detection of direct activity impractical.

II. THE LIFE-HISTORY OF THE TEST ORGANISM (*HETERAKIS SPUMOSA*)

The adult worms live in the caecum and colon, and eggs laid by the female worm require 14 days' incubation before the contained larvae are infective. The embryonated eggs are given via a stomach tube for artificial infections. The eggs hatch and larvae develop in the caecum, though a few may be found in the lower part of the ileum. With a mature infection some 70–80 % of the worms are found in the colon

with others in the caecum, and very few in the terminal portion of the small intestine.

Eggs appear in the faeces from 28 days after exposure to infection. The egg output of an infection induced by a single infective dose reaches its maximum between the 35th and 50th days. By the time the infection is 9 weeks old very few eggs are found in the faeces.

The contact of this worm with the tissues of its host is much less intimate than in the case of *Nippostrongylus muris*, and consequently little resistance is developed by the host. There is, therefore, no rapid fall in egg output or natural elimination of the worms as often occurs with *Nippostrongylus*, after 14 days' infection.

III. A COMPARISON OF THE TYPES OF ANTHELMINTIC TEST

Disregarding differences in the species of worms chosen as test infections there are four types of *in vivo* tests, the fourth of which is described here for the first time.

(1) *The worm egg-count method*—in which comparisons of worm egg counts are made before and after treatment of either natural or artificial infections. Reduction in the egg count is used to assess activity. This method is relatively inexact unless a consistently significant alteration in the worm egg count can be demonstrated. Numerous factors vitiate the accuracy of egg counts, thus, for example, when counts are based on weighed samples of faeces the great variation possible in the moisture content of the faeces seriously affects the resulting count. Secondly, immune reactions occur as can be shown experimentally with heavy infections of *Nippostrongylus* in which there is a naturally occurring and marked drop in egg output of the worms from the 12th to 14th days after infection. Subsequently, female worms may persist without laying. Thirdly, egg laying may be inhibited by low anthelmintic action without removal of the worms. This method, however, is of use in treating subjects which it is not wished to destroy for post-mortem examination. It is used with *Ascaris* or hookworm (*Uncinaria*) for assay purpose in dogs, and may also be used in 'pure genus' infections in sheep. Observations should be continued sufficiently long to exclude any reduction in egg count being due to inhibition of egg laying. Where several infections are present this method is of little value unless the different eggs can be accurately identified. This is seldom possible in ruminants or horses as there is considerable overlapping in size ranges of the eggs of the different species of worms. There is also a vastly different egg output with different genera of worms.

(2) *The critical anthelmintic test* (method of Hall) involves counting the worms removed by treatment which are found in the faeces in the 2–3 days after treatment, and also those remaining unexpelled at post-mortem examination which is made when excretion of worms has ceased. This evidence may be considered as 'direct' as it is unequivocal.

Critical testing was used by Hall and his colleagues on dogs and has resulted in the discovery of most of the anthelmintics in use for hookworms. This technique can only be used for relatively large worms and has not, so far as we know, been used in small animals (except as below).

(3) *Controlled anthelmintic tests* are performed with groups of treated animals which are compared with untreated controls. In these the activity detected may be considered as 'indirect' as it is based on a comparison of the worms remaining in the treated with those in the untreated controls. This is the usual form of chemotherapeutic test. Whitlock (1943) used the controlled method of testing comparing the worms remaining in untreated and experimentally treated groups. Rogers (1944) made egg counts on treated and untreated groups before and after treatment to forecast the worms to be expected in the treated by correlation of worms to eggs in untreated controls. By using comparisons of the worms in the two groups at post-mortem examination the same conclusions could be reached on Rogers' figures without the additional trouble of egg counting. Both methods have been tested here and the procedure of Whitlock is considered to be the more practical.

(4) *Controlled critical anthelmintic testing* in which both direct and indirect activity are detected. Controls are used to compare results in the treated groups with an untreated group ('indirect' activity). Counts are also made of the worms excreted to compare with the worms unexpelled by treatment (='direct activity'). The direct activity gives definite and unequivocal evidence of minor degrees of activity which would not be detected with certainty by indirect activity. For indirect activity to be regarded as significant a worm reduction of almost 50% is required in the treated as compared with the untreated group. The use of controlled critical testing has shown that the sum of the worms obtained from successfully treated animals (i.e. the worms removed by treatment and those remaining in the gut) is often less than the infection in the untreated group. This is particularly noticeable with certain groups of compounds which show a high indirect activity (by comparison with controls) and low or no direct activity (comparisons of worms excreted with total worm burden). The worms were probably affected by the compound and either digested and absorbed or destroyed by bacterial activity according to the part of the alimentary tract in which they existed. In many instances, therefore, the direct anthelmintic activity detected by critical testing is not the full anthelmintic activity. It is for these reasons that a controlled critical anthelmintic test is to be preferred as a routine sorting or comparative assay test for anthelmintics.

IV. TECHNIQUE

To prepare cultures for embryonation of the eggs of *Heterakis* female worms not less than 6 weeks old are finely mashed in 1% formol saline. The suspension of eggs is then filtered through a no. 120 sieve (120 meshes to the inch) and poured into Petri dishes to a depth of about $\frac{1}{3}$ in. for incubation at 26° C. for 14 days. At the end of this time the number of normally embryonated eggs is counted and an infective dose of approximately 250 embryonated eggs is given to each rat.

As the worms take 6 weeks to mature the smallest newly weaned rats are used when infecting for anthelmintic tests, as otherwise the rats are so heavy that they require unnecessarily large quantities of compound. Their average weight 6 weeks after infection is generally 100–110 g. with a range of 80–150 g.

The rats are used for testing anthelmintics when the worms are 41 days old.

Treatment is given after a 24–30 hr. fast, and the rats are killed 48 hr. after treatment. It is advantageous to fast the rats for 24 hr. before destruction. Each rat is kept in a separate cage from the time of treatment until they are killed. The faeces passed by each rat from treatment until death are kept and after soaking in water, the worms present are counted. At post-mortem examination the large intestine is removed, opened and scraped into a Petri dish containing water and the worms are counted.

(a) The development of worms in relation to infective dose

Of 301 rats exposed to 250 eggs of strain I of *Heterakis* the resulting infection averaged 39 worms, whilst with strain II an average of 96 worms developed in 120 rats. With an infective dose of 500 ova of strain I, 560 rats developed an average of 46·6 worms.

Table 1. *Development of worms in relation to infective dose*

Strain	Infective dose	No. of rats	No. of worms	Av. worms per rat	Percentage of worms developing
I	250	301	11,913	39	15·6
	500	560	26,081	47	9·4
II	250	120	11,550	96	38·4

(b) The excretion of worms in untreated rats

Observations have been made on several thousand rats infected with *Heterakis spumosa* for 4½–7 weeks.

The untreated groups on anthelmintic tests are dosed at the rate of 1 c.c. per 100 g. of the dispersing solution used for the dispersion of most of the compounds on test. This is without significant activity, and the excretion of worms is observed in these untreated rats for the 48 hr. after treatment and before their destruction. With strain I where the infective dose was 250 ova and the age of the infection was from 32–35 days (the great majority being in the range 32–39 days), the number of worms excreted amongst 301 rats infected with 11,913 worms was 209. Thus, there was an average normal excretion of 1·75 worms per 100. Only 1 in 5·5 rats excreted any worms, with an average excretion of 3·7 worms per rat excreting.

When the infective dose was 500 ova and the age of the infection as above, 545 worms were excreted amongst 560 rats infected with 26,081 worms. This equals an average normal excretion of 2 worms per 100 worms. Only 1 in 8 rats excreted worms with an average of 7·5 worms per rat excreting. With strain II of *Heterakis*, 120 rats infected with 11,550 worms (the age of infection being 43 days), excreted 60 worms. The average excretion was 0·5 % of the total worms. Only 1 in 7 rats excreted worms at an average of 3·5 worms each.

There is, therefore, a normal excretion of from 1·75 to 2 % for strain I and 0·5 % for strain II of *Heterakis spumosa*, as used. This requires consideration in the interpretation of direct activity and allowance is made where excretion is more than minimal. For details see Table 2.

Table 2. *Excretion of worms by normal untreated rats used as controls*

Strain	Infective dose	Age of infection in days	No. of rats	No. of rats excreting worms	Total no. of worms	No. of worms excreted	Percentage of worms excreted	Worm excretions of individual rats
I	500	32–56 (mostly 34–43)	560	73	26,081	545	2	108, 35, 27, 26, 25, 24, 22, 20, 11$^{\times 3}$, 10$^{\times 5}$, 8$^{\times 3}$, 7$^{\times 2}$, 6$^{\times 3}$, 5$^{\times 6}$, 4$^{\times 6}$, 3$^{\times 8}$, 2$^{\times 9}$, 1$^{\times 39}$
	250	34–55 (mostly 34–42)	301	55	11,913	209	1·75	28, 28, 24, 21, 4$^{\times 9}$, 3$^{\times 5}$, 2$^{\times 8}$, 1$^{\times 39}$
II	250	43	120	17	11,550	60	0·5	18, 11, 6, 4, 3, 2$^{\times 5}$, 1$^{\times 7}$

V. THE INTERPRETATION OF ANTHELMINTIC ACTIVITY

It is convenient to use percentages in the interpretation of the anthelmintic tests and these are calculated for the controlled test by the following formulae:

$$\text{indirect anthelmintic activity \%} = \frac{(\text{average worm count in untreated animals} - \text{worms surviving in treated ones}) \times 100}{\text{untreated worm count}}.$$

For example, if the worm count of the untreated animals is 400 and of the treated is 100, then the activity is 75%:

$$\frac{(400-100)\times 100}{400} = \frac{30{,}000}{400} = 75\%.$$

$$\text{Direct activity \%} = \frac{\text{worms expelled from treated animals} \times 100}{\text{worms expelled} + \text{worms surviving in treated animals}}.$$

'Indirect' activity, based on the comparison of treated and untreated control groups, is the usual method of interpretation adopted in chemotherapeutic techniques. Statistical examination has shown that for significance the indirect activity should be 50% on one test or may be less when confirmed by other tests. These observations were made using groups of eight treated animals and ten untreated controls.

'Direct' activity, which is based solely on observations in the treated animals is frequently lower than the indirect because a certain proportion of worms removed by anthelmintics are destroyed by enzymic or bacterial digestion.

Direct activity can, therefore, be regarded as minimal, and unequivocal so long as there is negligible excretion of worms in the untreated.

An example of the value of 'direct' activity in interpretation can be seen in Table 3 (Test 78) where the indirect activity of the 'lowest' dose of phenothiazine is quite insignificant but the direct activity is obvious.

The following definitions of various degrees of activity have been adopted for convenience:

Very active	Over 80% anthelmintic activity
Active	Over 60% anthelmintic activity
Moderately active	From 41–60% anthelmintic activity
Slightly active	From 30–40% anthelmintic activity

Where slight activity depends on indirect activity only, a repetition is desirable for confirmation. Less than 30% indirect activity with some evidence of direct activity may also qualify for the category 'slightly active', e.g. phenothiazine at 25 mg. in Table 3. The definitions of activity may be regarded as elastic.

Table 3. *The use of direct activity in interpretation*

Test 78.

Compound	Dose per 100 g. rat	Average worm counts: Gut	Average worm counts: Faeces	Individual worm counts	Percentage of activity: Indirect	Percentage of activity: Direct
Untreated (water)	1 c.c.	36	0	Gut: 38, 7, 51, 35, 38, 47 Faeces: 0, 0, 0, 0, 0, 0	0	0
Phenothiazine	25 mg.	32	12	Gut: 37, 14, 46, 15, 42, 27, 18, 51, 34 Faeces: 10, 1, 0, 13, 16, 30, 3, 6, 24	10	27
Phenothiazine	50 mg.	12	27	Gut: 6, 3, 25, 6, 1, 26, 2, 22, 18 Faeces: 8, 50, 26, 10, 54, 27, 32, 19, 21	64	69
Phenothiazine	100 mg.	8	47	Gut: 2, 15, 1, 5, 0, 8, 22, 0, 20 Faeces: 16, 18, 32, 75, 91, 39, 80, 33, 62	78	96

In the interpretation of activity consideration requires always to be given to the 'correctness' of the level of infection in the untreated group—this can sometimes be seen to be aberrant as, for example, when several of the treated groups have a higher worm burden than the untreated. In such cases adjustment of the controls to the average of the four highest infected treated groups may give a truer picture of the indirect activity. It can almost be postulated that where the indirect activity is markedly lower than the direct activity, such a result is erroneous. (It has already been emphasized that 'direct' activity can be regarded as minimal as a number of worms may be destroyed and escape detection.)

The examples in Table 4 show that adjustment of the untreated controls by correction to the average level of infection of the four highest treated groups gives a better approximation of indirect to direct activity.

Table 4. *The effect of adjusting control counts (untreated) to the average of the four highest treated groups*

Test no.	Percentage indirect activity on natural control counts	Percentage on adjusted control counts	Percentage direct activity
48	56	74	66
49	38	48	47
50	79	84	84
51	33	70	68
52	71	83	89
55	70	79	84
70	50	68	68
79	76	86	89

If it appears that the level of infection in the untreated group is higher than normal then interpretations of activity should be accepted with reserve as they may be too optimistic. As seen in Table 4, too low a control count gives too low a percentage for activity.

VI. THE RESPONSES OF THE TEST ORGANISM (*HETERAKIS SPUMOSA*) TO KNOWN ANTHELMINTICS

The chief anthelmintics acting on *Enterobius* are phenothiazine, which is not generally used in man on account of its occasional toxicity; gentian violet which has to be given in one or two courses of 20–30 doses and hexyl resorcinol. All these can be shown to have an activity against the test infection, though gentian violet is inactive in a single dose and has to be given in a series of doses near to the maximum tolerated. It is this relative inefficacy in man that imposes the necessity of a long course of treatment.

'Diphenan', about which convincing evidence concerning its activity against *Enterobius* is lacking, was found to be almost without activity.

The γ isomer of benzene hexachloride, which has been claimed by continental authors Klosa (1950) and Graeve & Herrnring (1951) as an active oxyuricide, shows only trace activity at the maximum tolerated single dosage. The latter authors were disappointed with the results obtained up to the time of publication.

Table 5. *The response of* Heterakis spumosa *to known oxyuricides*

Anthelmintic	Test no.	Dosage rate per kg.	No. of doses	Percentage of activity		Remarks
				Indirect	Direct	
Phenothiazine	Numerous tests (151 rats)	0·25 g.	1	38		—
Phenothiazine	Numerous tests (81 rats)	0·5 g.	1	53		—
Phenothiazine	Numerous tests (259 rats)	1 g.	1	73		—
Gentian violet	107	0·32 g.	16	17	3	All worms dead and shrivelled—activity higher
Gentian violet	118	0·15 g.	15	0	0	—
Hexyl resorcinol	61	0·2 g.	1	58	28	—
Hexyl resorcinol	200	0·1 g.	1	28	1	—
Hexyl resorcinol	200	0·2 g.	1	56	13	—
Diphenan	107	0·5 g.	12	35	12	Total dosage = 6 g./kg.
Diphenan	16	0·4 g.	5	0	0	—
Gammexane	200	40 mg.	20	12	6	Dosed b.i.d. for 2 spells of 5 days. 10 days in 14. Killed 16 days after 1st dose
Gammexane	200	25 mg.	20	5	5	Ditto. *Note*: Both dosage routines regarded as inactive

It seems reasonable to assume the reliability of this test for the detection of the activity of potential oxyuricides as the well-known and proven oxyuricides can all be detected and several other compounds with less well-known activity can also be recognized, e.g. some of the antihistamines which possess activity against *Enterobius*. For the detection of new anthelmintics (oxyuricide type) this test is parti-

cularly useful as on account of the sensitivity of the 'direct' activity interpretations minor degrees of anthelmintic activity can be detected with reasonable certainty. This is of considerable importance in the finding of chemotherapeutic 'leads' as the least active members of a series of chemical compounds are more likely to be tested first than the most active, which one hopes to find rationally. The indirect activity is of less value in this respect as it should approach 50% before being regarded as significant.

It can, however, also be used as an assay method for the quantitative comparison of several active compounds, and for this purpose it has been used extensively in an investigation into the improvement of existing anthelmintics such as phenothiazine. When used for the comparison of two samples it is advantageous to test a range of doses of each substance preferably with twofold increases over three or four doses. An example follows in Table 6.

Table 6. *The use of the test for assay purposes*

Dosage rate per 100 g. rat (mg.) ...	12·5	25	50	100	200
Preparation A					
Percentage anthelmintic activity	27 (6)	47 (32)	74 (39)	96 (7)	—
Preparation B					
Percentage anthelmintic activity	—	14 (6)	38 (25)	73 (26)	96 (7)

Figures in brackets = number of rats used; generally from 6–8 per test so at some levels 5 or 6 tests were undertaken.

The conclusion was that preparation A was twice as active as preparation B.

VII. DISCUSSION

The type of test described can be used with advantage to study many matters in the use of anthelmintics which are either in doubt or for which no satisfactory evidence exists. It is most difficult to obtain such definite evidence in field work with other than infections which behave in a straightforward manner. In fact, Walker, as recently as 1948, in discussing the chemotherapy of helminthiasis commented that, 'Research on the chemotherapy of helminthiasis has been hampered by the lack of suitable animals in which the infections can be studied'.

Using this technique it is now possible to make experimental comparisons with much more accuracy and ease than is possible in sheep or other similar animals. All the evidence available to date suggests that the compounds found most active in the laboratory test are also the most active in sheep.

From our observations in sheep (as yet unpublished) it appears that though the qualitative information derived from rats applies also to sheep, the quantitative differences may not be applicable. It is possible that the complicated digestive system of ruminants may actually assist the action of certain types of anthelmintics, particularly the insoluble ones like phenothiazine.

SUMMARY

An account is given of the technique and interpretation of a test which can be used for the detection of anthelmintics active against threadworm (*Enterobius*) in man and a number of the helminth infections in herbivorous animals.

It can also be used for the qualitative comparison of active compounds.

It has been used to investigate some of the fundamental practices in the use of anthelmintics—the results of which are to be published elsewhere.

I am pleased to acknowledge my gratitude to Miss M. Okell, Miss B. Bellamy, Miss J. Lonsdale and Miss H. G. Forster for their able technical assistance in the development and operation of this technique.

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ANTHELMINTIC STUDIES: II. A DOUBLE ENTERO-NEMACIDAL ANTHELMINTIC TEST COVERING A WIDE RANGE OF ACTIVITIES

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(With 1 Figure in the Text)

CONTENTS

PAGE

I. Introduction 242
The choice of infections—their zoological relationships and anthelmintic reactions 243
II. The use of *Nippostrongylus muris* as a test organism 243
(*a*) The life cycle 243
(*b*) The behaviour of *Nippostrongylus* and *Heterakis* as single and combined infections 245
(*c*) The technique of using *Nippostrongylus muris* for anthelmintic testing . 245
(*d*) The interpretation of activity 246
(*e*) The response of *Nippostrongylus muris* to known anthelmintics . . 246
(*f*) The effect of age of the infection on the result of anthelmintic treatment 249
III. The use of *Nippostrongylus muris* with *Heterakis spumosa* as a wider range anthelmintic test 249
(*a*) Details of use 249
(*b*) The responses of the combined infections to control treatments . . 249
(*c*) Definitions of activity in the double nemacidal test 249
(*d*) Routine details of organization 251
IV. Discussion 253
Summary 254
References 254

I. INTRODUCTION

In the routine screening of compounds for chemotherapeutic activity it is generally advisable to use as wide a range of infections as is practicable, but this is particularly so in the field of anthelmintics where such marked and strict specificity of action occurs.

For this reason when testing purely speculative compounds it is an advantage to use more than one infection, and the present paper deals with two infections combined in the rat. These are *Heterakis spumosa*, the use of which has been described elsewhere (Steward, 1955) and *Nippostrongylus muris* which has been used by Whitlock (1943) and Rogers (1944).

In this paper it is not proposed to discuss in full the details applying to *Heterakis* except in so far as relates to the other infection, *Nippostrongylus*, the use of which will be more fully described. The types of anthelmintic test that can be used were reviewed in the previous paper and this also will not be considered further.

The choice of infections—their zoological relationships and anthelmintic reactions

At the head of Table 1 are set out the familial relationships of the most important gastro-enteric parasites of man and animals. At the bottom of the table are the current treatments for these infections. Midway are the test infections which have been chosen for use, and these are linked by arrows with the infections whose anthelmintic responses are similar. Where the shaft of the arrow is discontinuous the response is only partial as, for example, in the relationship of the response of *Heterakis* to ascaricides. *Heterakis* responds regularly to hexyl resorcinol but only in about 50 % of tests to tetrachlorethylene which is more specific in its action against *Ascaris*. Similarly, *Nippostrongylus* in about 50 % of occasions responds to phenothiazine which is active in high doses against most of the related Trichostrongylid infections in sheep.

II. THE USE OF *NIPPOSTRONGYLUS MURIS* AS A TEST ORGANISM

(*a*) *The life cycle*

The eggs laid by *Nippostrongylus* are passed in the host's faeces and on incubation at a suitable temperature hatch, and in about 5 days develop into infective larvae. These infect by penetration of either the skin or, if swallowed, of the ali-

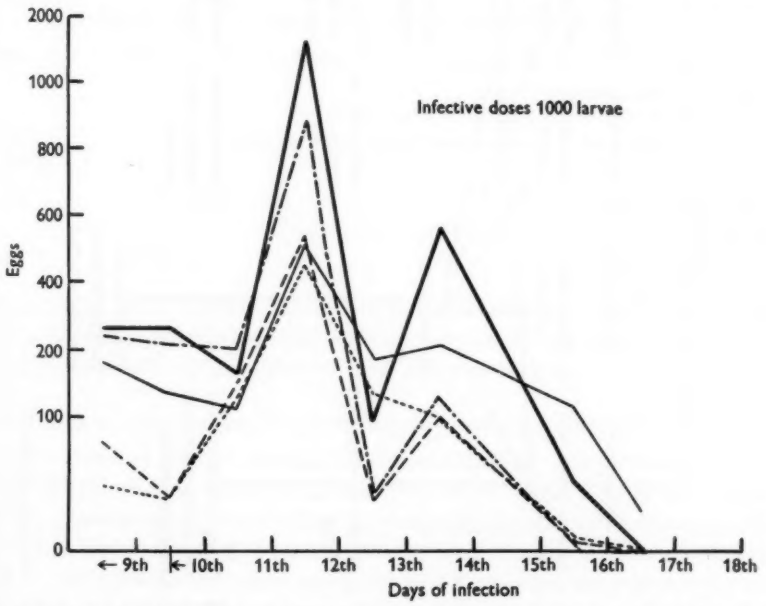
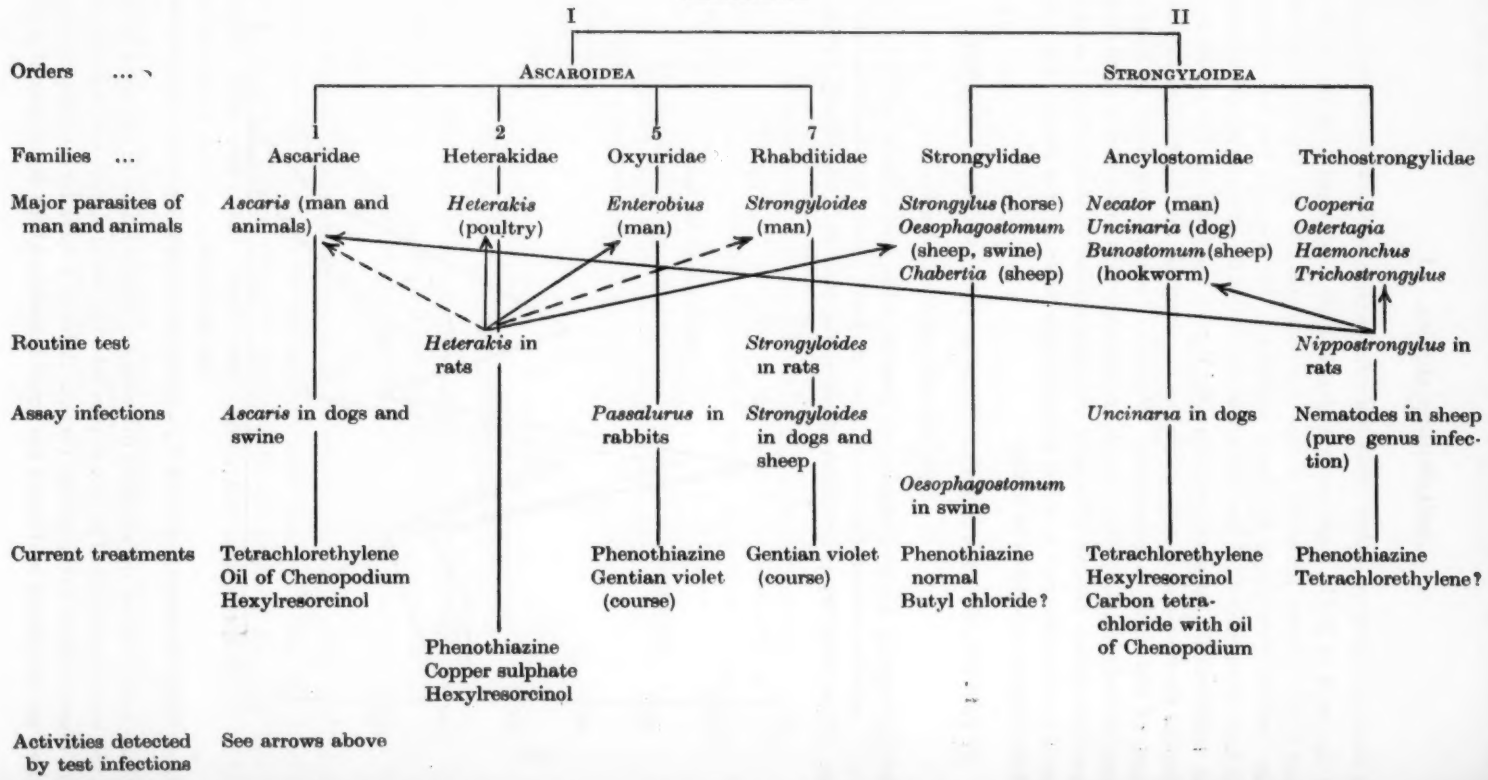


Fig. 1. Graph showing the level of *Nippostrongylus muris* infection in five albino rats.

mentary tract—to set up artificial infections a selected dose of infective larvae is injected subcutaneously. The larvae pass into the blood stream and migrate to the lungs where further development occurs. After 3–4 days larvae emigrate up the trachea, are swallowed and reach the small intestine where development is finally

Table 1

NEMATODA



– – – – → = activities partially or probably detected by test infection *Heterakis* (*Ascaris*, partial; *Strongyloides* not yet confirmed)

completed in the mucosa. The worms become mature by the 7th to 8th days and lay eggs from the 8th day onwards. Egg laying reaches a maximum between the 11th and 15th days after infection (Fig. 1). Through their close contact with the tissues and their blood migratory tour a fairly marked resistance develops to the worms. This is first detectable in heavy infections by the sharp fall in egg production occurring from about the 13th day onwards, by the 17th day the egg output may be nil. The next effect of the development of the host's resistance is actual elimination of the worms.

The degree of immunity that develops is apparently associated with the size of the infective dose.

It is, of course, necessary to test anthelmintics before natural excretion of the worms begins.

(*b*) *The behaviour of* Nippostrongylus *and* Heterakis *as single and combined infections*

At an early stage in our investigations, the effect of the infections used singly and together was compared, and it was found that neither of the two infections had any marked effect on the other.

In numerous subsequent tests there arose nothing to alter this conclusion.

Table 2. *Level of infection of single and double infections*

Test no.	Inf. dose	No. rats	Av. no. worms	No. rats	Av. no. worms
		Heterakis alone		*Heterakis* when combined with *Nippostrongylus*	
XII	250 ova	8	89	7	90
XIII	250 ova	10	81	9	76
		Nippostrongylus alone		*Nippostrongylus* when combined with *Heterakis*	
XIII	500 larvae	9	450	9	499

The figures in Table 2 relate to rats infected with 500 larvae of *Nippostrongylus* about the 32nd day after being infected with 250 ova of *Heterakis*. They were then killed 12–14 days after being infected with *Nippostrongylus*.

(*c*) *The technique of using* Nippostrongylus muris *for anthelmintic testing*

Rats are infected with a standard infective dose of *Nippostrongylus* larvae. Whitlock (1943) used an infective dose of 500 larvae whilst Rogers used 2000. It was found convenient for the present investigation to use 500 larvae, though at times when a lower 'take' of the infection was occurring the infective dose was increased to 1000. The infective dose was given in 0·2 c.c. water subcutaneously under the skin of the abdomen. The rats are treated on the 10th–12th day after infection and killed and examined 2–3 days later. The rats are fasted for 18–24 hr. before treatment and 24 hr. before destruction.

At post-mortem examination the small intestine is removed in one intact straight piece and syringed through with 20 c.c. water from a self-refilling syringe into a 4 oz. glass jar which is stored for 1 or 2 days in the refrigerator.

– – – → = activities partially or probably detected by test infection *Heterakis* (*Ascaris*, partial; *Strongyloides* not yet confirmed)

The washed out gut is compressed between two sheets of glass and the worms remaining are counted against the light. The bottles of gut washings are topped up with a solution of sodium bicarbonate to aid solution of mucus and after refrigeration the worms are counted using a lamp which provides convenient magnification and suitable illumination.

In making the count the sample is made up to a suitable volume mixed, and two samples of one-tenth are taken, and if the counts show no great discrepancy and the total worms counted comes to a total of not less than 40 the worm burden is calculated on the two samples which amount to one-fifth.

In low counts further samples are counted until at least 40 worms have been counted or the whole sample examined.

Where the counts of the samples are dissimilar further samples are counted until some uniformity is reached or the greater part of the sample counted.

(*d*) *The interpretation of activity*

The average worm counts of the treated group are compared with the average worm count of the untreated rats, and a successfully treated control group is kept to observe the response of each test to a standard control treatment (tetrachlorethylene or *n*-butyl chloride).

The percentage anthelmintic activity is calculated by the use of the following formula:

$$\% \text{ indirect activity} = \frac{\left(\begin{matrix}\text{average count of worms} \\ \text{in untreated group}\end{matrix} - \begin{matrix}\text{average count of worms} \\ \text{in treated group}\end{matrix}\right) \times 100}{\text{average worm count of untreated}}.$$

This type of activity may be regarded as 'indirect', and is based like most chemotherapeutic evidence on the comparison of treated with untreated groups. It differs from the 'direct' type of activity which is calculated on the proportion of worms actually excreted—such a form of calculation cannot be applied to *Nippostrongylus* which is a small worm and occurs relatively high in the alimentary tract so that it is destroyed and digested rather than excreted in a visible form in the faeces. Natural variations occur in the distribution of the worms between groups of rats as well as individuals though all possible steps to minimize such variations have been taken. Such steps included careful calculation and measurement of equal infective doses from well-mixed suspensions of larvae, and the use of groups of 8 to 10 inbred rats of uniform weight.

Numerous test results have been examined from a statistical angle by my colleague, Dr O. L. Davies, whose valued advice has been most helpful. The conclusion reached was that a 50 % reduction in worm count was required for significance. A lower reduction which was confirmed on repetition was regarded as partial activity. The definitions of activity are set out later (Table 8).

(*e*) *The response of* Nippostrongylus muris *to known anthelmintics*

The anthelmintics especially active against *Nippostrongylus muris* are of the fat solvent cuticle penetrating type such as tetrachlorethylene, *n*-butyl chloride. This may be because such anthelmintics penetrate the mucus in which the parasites

Table 3. *The response of* Nippostrongylus muris *to known anthelmintics—some test results*

Compound	Dose per 100 g. (c.c.)	Test	Av. worm count of untreated	Av. worm count treated	Activity (%)
Tetrachlorethylene	0·2	3	462	76	84
	0·2	—	496	6	99
	1	7	482	33	93
	1	5	512	101	80
	0·03	3	462	368	20
	0·03	2	496	500	0
	0·1	V	115	3	97
	0·1	73	309	15	95
	0·1	69	333	52	85
	0·1	67	267	62	79
	0·1	66	303	55	82
	0·1	65	352	81	77
	0·1	61	170	50	70
	0·1	59	97	9	91
	0·1	58	135	6	95
	0·1	57	149	61	60
	0·1	56	181	31	82
	0·1	55	116	47	59
	0·1	54	310	135	57
	0·1	52	120*	144	0 or 31*
n-Butyl chloride	0·2	18	251	98	61
	0·2	19	59	10	83
	0·2	17	197	5	98
	0·1	V	115	48	67
	0·2		115	7	94
	0·1	IX	1163	567	52
	0·2		1163	263	77
	(mg.)				
Phenothiazine	200	16A	989	382	61
	200	16B	259	291	0
	200	16C	841	491	42
	200	16D	213	207	3
	100	7	482	462	4
	100	5	512	487	5
	100	4	498	346	10
	100	94	226	186	17
	100	73	309	192	37
	100	69	333	177	49
	100	67	267	255	4
	100	66	303	320	0
	100	61	170	86	49
	100	49	97	58	40
	100	49	135	77	43
	100	47	149	117	22

* Three other groups av. 210; control infection therefore low and if adjusted to 210 activity would equal 31 %.

lie more effectively than other types of compounds. Rogers (1944) observed that both carbon tetrachloride and tetrachlorethylene stimulated *Nippostrongylus* to leave mucus. As these worms reside in the small intestine through which passage occurs relatively rapidly the successful anthelmintic must act quickly.

In Table 3 are typical examples of the response of *Nippostrongylus* to various dosages of tetrachlorethylene, *n*-butyl chloride and phenothiazine.

A dosage rate of 0·1 c.c. tetrachlorethylene/100 g. rat is almost always active. An exception may be test 52 in which the treated group had more worms than the untreated (144 to 120) but examination of other (unsuccessfully) treated groups showed three with averages of 210, 210 and 211 worms per group. If the control group were adjusted to 210 then tetrachlorethylene would have an activity of 31 %. In this test, however, the diluted sample of tetrachlorethylene was a week old and may have deteriorated. *n*-Butyl chloride is rather less active than tetrachlorethylene on an effect/dosage basis.

Table 4. *Summary of results of various doses of phenothiazine on* Nippostrongylus muris

Dose per 100 g. rat (mg.)	No. of rats	Average worm count		Indirect activity	
		Untreated	Treated	%	Range
50	12	293	260	10	(6–13)
100	50	372	283	24	(0–74)
200	69	378	295	22	(0–74)

n-Butyl bromide appears more active than *n*-butyl chloride (Table 5).

Table 5. *Test XXXVIII. A comparison of* n-*butyl chloride with* n-*butyl bromide*

(Details: *Nippostrongylus muris* inoculum 500 larvae, 6 days' water. Rats treated on 11th day and killed on 13th day of infection.)

Compound	Dosage per 100 g. rat (c.c.)	No. rats surviving	Average worm count	Activity (%)
Untreated	—	8/8	230	—
Tetrachlorethylene	0·1	4/6	154	33
n-Butyl chloride	0·2	5/6	51	78
n-Butyl bromide	0·2	4/6	3	99
n-Butyl chloride	0·1	6/6	48	79
n-Butyl bromide	0·1	6/6	27	88

Phenothiazine shows an activity which is quite variable. The test results in Tables 3 and 4 are by no means complete but in the twelve tests (at 100 mg./100 g. rat) it is recorded that (Table 3) activity ranges as follows:

0, 4, 4, 5, 10, 17, 22, 37, 40, 43, 49 and 49 %.

On seven occasions activity was below 35 % and on five it was between 37 and 49 %. Table 4 gives an average of a series of tests. Rogers attributes the lack of activity of phenothiazine that he observed against *Nippostrongylus* to its rapid passage through the small intestine of rats. This is much quicker than in sheep in which phenothiazine is active against several Trichostrongylid worms related to *Nippostrongylus*. The results recorded above indicate that phenothiazine is active, though variably, against *Nippostrongylus* in rats. Its variability against Tricho-

strongylids in the small intestine of ruminants is also well known and there are several reports that very high doses are required for its effect to be high (Gibson, 1945; Steward, 1950).

(f) The effect of age of the infection on the result of anthelmintic treatment

There is some field evidence that immature infections are less responsive to treatment than mature, and definite evidence on this point was obtained when an experiment was undertaken to determine the best age of the infection for optimum anthelmintic activity.

Table 6 gives the details of one test (XXV) with treatments on different days.

Table 6. *The effect of age of the infection on response to treatment*

(Test XXV: inoculum 1000 larvae (stored for 10 days before use). Rats treated on 3rd, 6th and 10th days and killed on 12th day of infection.)

Treatment	Dose per 100 g. (c.c.)	Day of dosing	No. of rats surviving treatment	Average worm count	Activity (%)
Untreated	—	—	10/10	304	—
n-Butyl chloride	0·2	3rd	8/10	181	40
n-Butyl chloride	0·2	6th	7/10	65	79
n-Butyl chloride	0·1	10th	10/10	41	86

III. THE USE OF *NIPPOSTRONGYLUS MURIS* WITH *HETERAKIS SPUMOSA* AS A WIDER RANGE ANTHELMINTIC TEST

(a) Details of use

Details of the use of *Heterakis* as a controlled critical nemacidal test have already been described (Steward, 1955).

When it is wished to combine the two infections it is convenient to superimpose *Nippostrongylus* on the other by inoculation of the infective larvae when the *Heterakis* infection is 3½–4½ weeks' old so that the *Nippostrongylus* infection is 10–12 days' old and *Heterakis* 34–42 days' old when the treatment is applied.

(b) The responses of the combined infections to control treatments

The responses of the combined infections to the control anthelmintic treatments do not differ from the single but the attempt to use a combination of the two control treatments for the double infection gave results which indicate that reduced anthelmintic effect can generally be expected from combination of the two treatments (see Table 7).

(c) Definitions of activity in the double nemacidal test

The calculation of the percentage of anthelmintic activity is made on the following formulae:

Direct activity (*Heterakis* infection only)

$$= \frac{\text{average count of worms expelled} \times 100}{\text{average count of worms expelled} + \text{worms surviving in treated group}}.$$

Indirect activity (both infections)

$$= \frac{\text{average count of worms in untreated} - \text{worms surviving in treated group} \times 100}{\text{untreated worm count}}.$$

Table 7. *Effect of combined control treatments on* Heterakis spumosa *and* Nippostrongylus muris

Test no.	Compound	Dose per 100 g. rat	No. of rats	*Nippostrongylus* Average worm counts		Indirect activity (%)	*Heterakis* Average worm counts				Activity (%)	
							Untreated		Total			
				Untreated	Treated		Gut	Faeces	Gut	Faeces	Indirect	Direct
Nippostrongylus XXIV	*n*-Butyl chloride	0·2 c.c.	6	295	3	99	—	—	—	—	—	—
	n-Butyl chloride + phenothiazine	0·2 c.c. + 200 mg.	10	295	56	81	—	—	—	—	—	—
Nippostrongylus XXIV	Tetrachlorethylene	0·1 c.c.	10	295	20	93	—	—	—	—	—	—
	Tetrachlorethylene + phenothiazine	0·1 c.c. + 200 mg.	7	295	151	49	—	—	—	—	—	—
Nemacidal III	Tetrachlorethylene	0·1 c.c.	8	454	174	62	57	—	31	—	46	—
	Phenothiazine	200 mg.	10	454	472	0	57	—	6	—	90	—
	Tetrachlorethylene + phenothiazine	0·1 c.c. + 200 mg.	10	454	26	94	57	—	21	—	63	—
Nemacidal IV	Tetrachlorethylene	0·1 c.c.	6	227	10	96	51	0	36	13	28	26
	Phenothiazine	200 mg.	8	227	95	58	51	0	11	46	70	81
	Tetrachlorethylene + phenothiazine	0·1 c.c. + 200 mg.	7	227	33	86	51	0	20	62	44	76
Nemacidal IX	Phenothiazine	200 mg.	8	541	521	4	14	0	3	3	79	50
	Phenothiazine + tetrachlorethylene	200 mg. + 0·1 c.c.	5	541	100	82	14	0	2	1	86	33

For convenience definitions of various degrees of activity have been adopted and these are set out in Table 8.

In calculating the percentage of activity the formulae are used according to rule, but in placing a compound in a class of activity some latitude may be taken and allowance made for the level of infection in the untreated controls which may on occasion be seen to be low or high in relation to the level of infection of a few unsuccessfully treated groups. In the former case the activity is unflattering and the latter flattering to the compounds under test.

Table 8. *The interpretation of activity in the double nemacidal test*

Activity (%)	Interpretation	
	Heterakis	*Nippostrongylus*
Over 80	Very active	Very active
Over 60	Active	Active
41–60	Moderate activity	(50–60 %) = moderate
30–40	Slight activity	(30–50 %) if repeated = slight activity
Less than 30 % if some direct activity	Less than 30 % indirect but some direct activity is recognized as definite ('Slight activity')	Below 30 % = inactive

(*d*) *Routine details of organization*

It may be advantageous to mention a few details of the organization as quite large numbers of animals can be dealt with without difficulty if some attention is paid to the details of procedure. At the commencement of the test the rats are grouped on weight and placed in individual cages (drawers of a metal filing rack). Each rat is kept separate from all others from the time of grouping until death and on the cage are card labels, in duplicate, with the group and individual rat number recorded. Weights are entered on a pad at the time of dosing, and later in a book. Each rat has a removable wire grille floor through which faeces drop to the floor of the cage (drawer).

When the rats are killed one label is affixed to its tail by pulling the tail through a pencil hole in the card and the other label is placed in a 4 oz. wide-necked bottle in which all the faeces from the cage are placed for examination later. When all have been collected all the bottles have hot water added to the faeces.

The dead rats are sent on trays from the animal room up to the laboratory where the post-mortem examinations are made. With five assistants available it was found convenient to allocate the duties as follows:

Assistant no. 1—seated at the extreme left—has the trays of dead rats within reach, each with a label on its tail. She lays out the rat on an automatic post-mortem board, opens the abdomen and removes the whole of the intestine intact in two pieces—the small and large intestines separately, placing them with the label in a Petri dish. The label which was one of the two removed from the rat's individual cage has the rat's group and individual number on it, and no. 1 writes on the label the sex of the rat which is later entered by no. 5 on a rough pad for

transcription into the permanent record book together with the count of the worms in the large intestine (*Heterakis*).

Assistant no. 2—seated to the right of no. 1—receives the Petri dish, retains the small intestine and passes the large intestine to assistant no. 4. A jar is ready numbered with the rat's number. Assistant no. 2 places the duodenal end of the small intestine over a blunt needle attached to an automatically refilling 20 c.c. syringe, and washes it through with two syringe-fulls of water into the labelled bottle. The washed gut is then squeezed out into the bottle and then it is passed to assistant no. 3. The bottles are dealt with later.

Assistant no. 3 straightens out the small intestine on a sheet of glass, superimposes another sheet of glass and compresses them together so that any worms which have not been washed out by no. 2 can be seen against the light through the wall of the gut. These are counted and recorded on a rough pad. The small intestine is then discarded—still unopened.

Assistant no. 4, who received the whole of the large intestine in a Petri dish from no. 2, adds water to the dish and then with angular scissors (one point blunt) opens the whole of the large intestine and with the angular back of the scissors scrapes the mucosa under the water in the dish. The dish, with card still present, is passed to no. 5—the opened gut having been discarded.

Assistant no. 5, with the aid of a dissecting needle to move debris, counts the worms present and records them together with the sex of the rat against its group and individual number. Any abnormality of colour or consistency of the faeces or staining of worms in the treated groups is recorded at this stage.

The bottles containing the Nippostrongyles washed out with about 40 c.c. water by assistant no. 2 are filled to about 100 c.c. level with a saturated solution of sodium bicarbonate to clear the mucus, etc. They are placed in a refrigerator for 24 hr. and counted later by no. 2 assisted by no. 3.

The contents of each bottle are poured into a cylinder which is topped to a convenient volume, 100 c.c. This is poured into a jar, mixed, and two samples of one-fifth the volume are withdrawn with a large syringe and expelled into two Petri dishes. The worms in these samples are counted by assistant no. 2 and recorded. If no great discrepancy is found between the counts and the total worms counted are not less than 40, the counts are entered on a pad, averaged and later transcribed into the permanent note book. Otherwise, further samples are counted as described earlier.

The counts of *Nippostrongylus* are made with the aid of cooled illumination and magnification using a lamp known as an 'illuminated industrial magnifier'.*

The samples are counted in a flat-bottomed square glass or Perspex dish ruled in blue squares over a white background for *Nippostrongylus* as the worms are red, and over a black for *Heterakis* as the worms are white. Magnification is unnecessary for *Heterakis*. The faeces which have been left to soak in warm water overnight are examined for the number of *Heterakis* excreted in each rat's faeces.

* Manufactured by the Engineering Development (England) Co., 50 Pall Mall, London. It is provided with an aspheric lens 5 in. in diameter giving a magnification $\times 2$ with a working distance between the lens and the object of 5 in. and the eye of the lens of 6 in.

IV. DISCUSSION

The double nemacidal test is designed as a screening test for speculative anthelmintics about which nothing may be known. Compounds of chemical groups known to possess activity against one type of infection would naturally be tested against the relevant infection.

It can also be used to assess alterations in anthelmintic potency that follow variations in the formulation of an anthelmintic, which variations may be made either empirically or rationally. Some of the advantages may be obvious such as the avoidance of duplication of the use of animals and quantities of valuable chemical compounds and many steps in the testing technique which can be saved by the duplication of the infections used. The simple matter of animal accommodation and cages is worth consideration where large numbers of animals have to be kept 4 or 5 weeks for an infection to mature.

Statistical examinations and experience are in close agreement that in interpreting 'indirect activity' there should be a reduction in the average worm burden of the treated group of about 50 %, and this applies where the number of animals in a group is seven or eight. A much lower 'direct activity' can, however, be accepted with reasonable safety so long as due attention is paid to the state of infection in the control and other treated groups.

Much evidence has accumulated to show that there is a marked tendency for great variation in effect to occur with incompletely effective doses of almost any anthelmintic. It has been a common finding that even with reasonably effective doses of say, for example, phenothiazine, that of ten rats treated with the same dosage rate that some will be completely cured, some partly and some may be almost unaffected.

If such variation occurs within a group it can naturally be understood that once in a while a whole group may act aberrantly. An incomplete example of this tendency occurs in Table 3, test 52, with 0·1 c.c. tetrachlorethylene dosage against *Nippostrongylus*. Here when adjustment is made for a low control group the activity only amounts to 31 %, whereas normally it seldom shows less than 70 % activity.

As almost all the anthelmintics active against *Enterobius*, Strongyles (*Oesophagostomum*, *Chabertia*, *Strongylus*, etc.), Hookworms (*Necator*, *Ancylostoma*, *Uncinaria*), Trichostrongyles (*Haemonchus*, *Trichostrongylus*, etc.) and *Ascaris* can be shown to be active against one or other of the test infections used it appears rational to expect that most compounds active against these genera will be detectable by this test.

As a strict requirement for an effective anthelmintic is that it should act in a single dose the test has only been used with a single high dose of the speculative compounds tested. In this connexion, the technique of Brackett & Bliznick (1949) involved the feeding of drug diets to mice infected with *Nippostrongylus* but by their technique even such an effective anthelmintic as tetrachlorethylene was more or less without effect. One wonders what would be the practical value of even an effective drug that had to be administered over a long period in the diet.

SUMMARY

A technique is described of the use of two infections in the rat which respond to the anthelmintics acting on a very wide range of gastro-intestinal nematodes. It can be used as a routine screening test for anthelmintics or for the study of variations in the degree of activity caused by alterations in formulation or presentation.

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ANTHELMINTIC STUDIES: III. A TAENIACIDAL TESTING TECHNIQUE

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CONTENTS

	PAGE
I. Introduction	255
(*a*) Existing techniques	256
(*b*) Choice of test infection	257
II. The life-cycle of *Hymenolepis nana* var. *fraterna*	257
III. The technique of taeniacidal testing using *Hymenolepis nana* in mice	259
IV. The interpretation of taeniacidal activity	261
(1) Interpretation of the test	261
(2) The use of a form of computation	261
(3) Definitions of activity	261
(4) The use of controls	262
V. The response of the test organism to known taeniacides	263
VI. The requirements of a good anthelmintic for tapeworms	264
VII. Discussion	265
Summary	265
References	265

I. INTRODUCTION

Some of the existing modes of treatment for tapeworm that are still used in man and animals have been discovered as a result of the sagacity of native medicine men. Areca nut (arecoline is widely used in dogs), pumpkin seeds, pelletierine, male fern and kamala are substances all of which probably came into use from native medicine.

Mepacrine was found active in mice infected with hymenolepid tapeworms by Culbertson (1940) and was successfully used against *Hymenolepis nana* in man by Berberian (1946). It has subsequently been found variably effective against *Taenia saginata* in man by several workers (Mazzotti, Rodriguez & Treviño, 1947; Pipkin & Risk, 1948).

These modes of treatment can all be regarded as having been found more or less empirically.

Several compounds have been found effective by primary testing in dogs and these include *iso*-amyl-ortho-cresol which is more active against *Dipylidium* than the *Taenia* species (Stewart, 1937–8); perthiocyanic acid which may be too toxic for field use, Enzie (1944), and 2,2′-dihydroxy-5, 5–dichlorodiphenyl methane (Craige & Kleckner, 1946).

The current treatment with copper sulphate for tapeworm in ruminants was discovered in its use against a nematode infection (*Haemonchus*).

No effective treatment for the various tapeworms in poultry is in general use in this country, though tapeworm is a serious infection of adult and growing birds.

In Northern Ireland where the poultry industry is so large the disease is considered of major importance. In the U.S.A. tin oleate and synthetic pelletierine hydrochloride, or tin oleate with triethanolamine or ammonium hydroxide were used successfully in limited trials by Guthrie & Harwood (1944) on experimental tapeworm (*Raillietina* sp.) infection of chickens. There is no evidence of its being used in the field. In 1947, Guthrie secured a U.S. patent covering a mixture of stannous maleate and ferric arsenate for the treatment of tapeworm in poultry.

These taeniacides in poultry appear to be the only ones which have been discovered in a routine search which was conducted by Harwood and Guthrie *et al.* (*vide infra*).

It is only when deliberate search has been made with a suitable technique operated on a large enough scale that the most effective modes of treatment will be discovered.

(*a*) *Existing techniques*

The actions of drugs on Cestodes *in vitro* were studied by Duguid & Heathcote (1950) who reviewed previous similar studies. They found that there were marked differences between the action of drugs in earthworms and tapeworms *in vitro* and performed their observations on portions of *Moniezia expansa*, the sheep tapeworm.

da Costa (1930, 1932) in Lisbon made kymographic records of drug actions on segments of *Taenia serrata* or *Dipylidium caninum*.

Harwood & Jerstad (1938) used a critical testing technique with chickens artificially infected with a single cyst of *Raillietina cesticillus*, dissected from infected meal beetles (*Tribolium* spp.). They were seeking a successful treatment for tapeworm in chickens and perhaps naturally chose the chicken as the test animal and *Raillietina* as the test infection. Several publications on preparations (tin compounds and miscellaneous substances) tested against this tapeworm have appeared in the last few years, Harwood & Guthrie (1940), Guthrie, Powick & Baudel (1941) and Guthrie & Harwood (1941, 1944).

Holton (1947) used a mixed natural infection of *Hymenolepis diminuta* and *H. nana* in mice, the former infection predominating. Two hours after treatment the mice were purged with magnesium sulphate and 3 hr. later the faeces were examined for *Hymenolepis* fragments which were collected and weighed. The mice were killed and worms from each group of mice divided into three categories which were weighed separately, namely:

(1) Worms from the large intestine (not normally inhabited by *Hymenolepis*).

(2) 'Dead' worms from the small intestine.

(3) Live worms from the small intestine.

The weight of the affected worms (categories (1) and (2) and worms in the faeces) was expressed as a percentage of the total weight of worms.

This technique may be well designed for the comparison of active substances, but as a sorting technique for taeniacides possesses several disadvantages.

First, the worm on which most of the evidence was obtained, *H. diminuta*, which is very much larger than *H. nana*, differs from the most important pathogenic tapeworms in that it has an 'unarmed' scolex with less power of fixation than most species.

Secondly, the period of observation is not long enough to detect the action of taeniacides which act slowly as, for example, Mepacrine.

Thirdly, the infections being 'naturally' acquired are likely to be of different age-groups which is obviously a disadvantage in chemotherapy. It appears likely to be more troublesome to operate as a screening test (for which purpose it was probably not designed) than the technique described below.

(*b*) *Choice of test infection*

The selection of *H. nana* var. *fraterna* as the test infection is based on the fact that it is almost the only tapeworm which can be transmitted directly without an intermediate host but in other respects is similar to most pathogenic tapeworms ('armed' scolex, etc.). It is also well adapted to mice which are themselves a suitable experimental host and convenient for use in chemotherapy. The strain of *H. nana* used in the experiments described below was obtained from naturally infected laboratory white mice purchased from outside sources.

Table 1 shows the relationships of the more important cestode parasites of man and animals, together with the test infection and the anthelmintic substances in current use.

II. THE LIFE-CYCLE OF *HYMENOLEPIS NANA* VAR. *FRATERNA*

The eggs of this tapeworm may be passed in the faeces or liberated when detached segments of the worm break up. They are infective when passed and if swallowed by a susceptible host the hexacanth embryo bores into the mucosa of the upper part of the small intestine where it develops into the cysticercoid—mostly in the second quarter of the gut. The cysticercoids break out after 4½ days and have mostly emerged by 6 days after infection. Considerable resistance develops to super-infection as early as 4 days after exposure to the first infection. Growth takes place rapidly during the later days of the fixation of the head and from 14 days after exposure to infection eggs may be passed in the faeces. The development of many immature worms appears inhibited by the presence of mature worms and when these are removed others develop to maturity.

The adult worms occur mostly in the last quarter of the small intestine. There is natural excretion of the adults and most worms are excreted in 30–40 days. Resistance to infection increases with age; fewer animals become infected and fewer worms develop.

As well as the direct method of development described above cysticercoids can develop in the larvae of meal worms and other Coleopterous insects.

In the direct life-cycle the position of the 'intermediate host' is taken by the upper part of the small intestine. Auto-infection may be prevented by the position of the adult worms in the lower portion of the small intestine, but there is in any case marked immunity to re-infection set up by the development of the cysticercoid stage in the intestinal mucosa. Intensive studies on the host parasite relationships of this worm have been made by Hunninen (1935, 1936) and Joyeux (1925).

Table 1. *The zoological and anthelmintic relationships of the more important tapeworms of man and animals and the test infection*

COESTODA

	Order CYCLOPHYLLIDEA				Order PSEUDOPHYLLIDAE
Family ...	Anoplocephalidae	Davaineidae	Hymenolepidae	Taeniidae	Diphyllobothriidae
Genera	*Moniezia* Cattle Sheep	1. *Davainea* Poultry 2. *Raillietina* Poultry	1. *Dipylidium* Dog 2. *Amoebotaenia* Poultry 3. *Choanotaenia* Poultry 4. *Hymenolepis* Poultry	*Taenia* Man and dog	*Diphyllobothrium* Man and dog
Routine test infection Intermediate host		*Hymenolepis* in mice Direct			
Assay infection		Relevant natural infections	or	Artificial infections *Taenia* spp. in dogs	
Intermediate host	Oribarid mites	Insects 1. Snails and slugs 2. Ants and beetles	Insects 1. Fleas or lice 2. Earthworms 3. Flies and beetles 4. Beetles, etc.	Beef, Pork } Man Rabbit, Sheep } Dog	Cyclops and Fish
Treatment			*Mepacrine	Man	
			*Tetrachlorethylene	Man	
			*Oleoresin aspidii	Man	
			Arecoline	Dog	
			*Dihydroxydichloro-diphenylmethane	Dog	
			†Copper sulphate	Ruminants	
			‡Lead arsenate	Sheep	

* This treatment is regularly effective against *Hymenolepis nana*.
† This treatment is sometimes definitely effective against *Hymenolepis nana*.
‡ Has not been tested against *Hymenolepis nana*.
Arecoline does not appear active against *Hymenolepis nana*—a taeniafuge.

III. THE TECHNIQUE OF TAENIACIDAL TESTING USING *HYMENOLEPIS NANA* IN MICE

As older mice may already be infected and as immunity increases with age the mice are used for infection at early weaning when they weigh about 12 g.

Fresh *Hymenolepis* eggs are obtained by mincing adult worms that are approximately 16 days old; batches of donor mice having been especially infected to provide the infective material. The mashed worms are passed through a 120-mesh sieve to remove the larger particles. A count is then made of the normal eggs and appropriate dilution made so that 200 normal eggs in 0·5 c.c. liquid are given to each mouse.

In 13 days' time the mice are grouped in tens with uniform distribution of heavy and light mice in each group. They are then fasted for 24 hr. and treated on the 14th day of infection. After grouping each mouse is put in a separate cage, in a rack of metal filing cabinet drawers each measuring $12 \times 5 \times 4\frac{1}{2}$ in. A battery containing sixty such cages measures $73 \times 28 \times 12$ in.

One group of ten mice is kept as an untreated control group and another is treated with an effective compound as a control for the response of the infection to a known treatment. The other groups when used for routine taeniacidal detection are treated with speculative compounds at a dosage rate of 1 g./kg., or the maximum tolerated dose if it is less than 1 g./kg. Fully active compounds are retested at lower doses.

The compounds are usually administered as solutions or dispersions in a mixture of dispersing agents ('Dispersols LN and OG') in 1 c.c. volumes per 20 g. mouse. Some compounds are dissolved in a small quantity of alcohol and dispersol is then added. All insoluble compounds are milled for 90 min.

The mice are purged with magnesium sulphate at 2 and 24 hr. after treatment in case any compounds with incomplete taeniafugal effect are on test. There is no evidence yet that this purge is advantageous and this step of procedure could probably be omitted without disadvantage as all the compounds found active have possessed taeniacidal rather than taeniafugal properties. The dose of purgative used appears to have no action against the infection (see Table 2).

The mice are fasted for 24 hr. before they are killed on the third day after treatment. As the mice are killed their ticket numbers are affixed to them (by making a hole in the card and pulling the tail through) and trays of the dead mice are sent up from the experimental room to the laboratory for worm counts.

The dead mice are laid out (for expeditious handling) on a special post-mortem board and the small intestine removed in one straight piece. The small intestine is then placed in a Petri dish with the mouse's ticket and a second assistant washes through the gut with 20 c.c. water from an automatic self-refilling syringe fitted with a blunt metal catheter (size 'O' hypodermic needle) or with an ordinary 20 c.c. syringe. If a perforation occurs in the gut through which the water under pressure escapes the gut is pulled on to the catheter beyond the puncture and washed out forcibly.

Table 2. *The effect of magnesium sulphate* (0·5 *c.c.* 13 % *solution*) *on* Hymenolepis nana *in mice*

	Dose per mouse	No. of mice surviving	No. of mice negative	Average computations		Mouse no. 1	2	3	4	5	6	7	8	Total	Total computations	No. of worms excreted in faeces
Untreated	—	8/8	1/8	85	VL	3	5	—	—	4	6	—	2	20	400	9 (5 small and 4 portions)
					L	5	1	1	2	3	1	—	1	14	140	
					M	8	1	1	8	4	—	—	5	27	135	
					S	—	—	1	—	—	—	—	—	1	1	
					VS	—	—	—	—	—	—	—	—	—	—	
Magnesium sulphate 13 % solution	0·5 c.c.	8/8	0/8	80	VL	1	—	9	1	1	3	—	—	15	300	6 (6 small)
					L	—	—	2	5	2	3	6	13	31	310	
					M	1	—	—	2	—	2	—	—	5	25	
					S	1	2	—	—	—	—	—	—	3	1·5	
					VS	—	—	—	—	—	—	—	—	—	—	

VL = very large; L = large; M = medium; S = small; VS = very small.

A third assistant counts the worms classifying them into five categories of different sizes and entering details on a rough note pad from which they are entered in a permanent record book.

IV. THE INTERPRETATION OF TAENIACIDAL ACTIVITY

(1) *Interpretation of the test*

Strong resistance is conferred by the infection following the development of the cysticercoids in the mucosa of the small intestine (Hearin, 1941). This resistance, which may last for 3 months, may account for inability to secure a 100 % infection as the mice used are obtained from outside sources.

There is a variable 'take' of the tapeworms in the mice which accept the infection and there is generally an inverse relationship between the numbers and size of the worms present. Thus, if a few worms are present they are probably large or very large, but if very many they will mostly or all be very small. This was observed by Chandler (1939).

There is also a tendency for partially effective anthelmintics to remove only the larger tapeworms and these two observations have led to our adoption of a method of computation to detect activity where the effect of the treatment was not complete.

(2) *The use of a form of computation*

Ordinarily, one can compare the number of mice containing no tapeworms in the treated groups with the number in the untreated groups. Often, for the reason mentioned above, an incomplete effect may not be clear except by using the method of computation. By this method, scores are allocated to worms of the different sizes as follows:

Very large	20	Actual sizes are	$> 1\frac{1}{4}$ in.
Large	10	Actual size about	1 in.
Medium	5	Actual size about	$\frac{1}{2}$ in.
Small	$\frac{1}{2}$	Actual size about	$\frac{1}{4}$ in.
Very small	$\frac{1}{10}$	Actual sizes are	$< \frac{1}{8}$ in.

These 'scores' have served without need to alter them over a large number of tests.

We have divided our interpretations into a number of classes of activity based on the removal of the worms, i.e. proportion of negative treated to negative untreated mice, and, where the activity is not complete, taking account of the computation. The computation is based upon the average score of infection for all surviving mice in the group and in addition those dying at 48 hr. or more after treatment.

(3) *Definitions of activity*

For convenience of interpretation compounds are classified into categories of different degrees of activity as follows:

Very active	75–99 % anthelmintic activity
Active	50–74 % anthelmintic activity
Slightly active	34–49 % anthelmintic activity
Possibly active	25–33 % anthelmintic activity

The lowest category of activity is accepted only on confirmation when repeated.

The classification of compounds into different categories of activity involves the use of the proportion of negatives in the treated as compared with the untreated mice together with the computations for the same groups. Rather more attention is usually paid to the negatives than the computation unless the latter is markedly reduced. The interpretations of a typical test (no. 137) are attached in Table 3. The compounds are designated by numbers—it is intended to report the actual results of testing at a later date.

Table 3. *Taeniacidal test* 137

Compound	Dosage per 20 g. mouse (mg.)	No. of mice surviving	Treated/untreated ratios		Conclusions
			Negatives	Average computations	
5770/5	1	10/10	9/10 to 1/10	3/45	Very active (control)
8348	20	10/10	4/10 to 1/10	48/45	Slightly active
8344	20	10/10	3/10 to 1/10	25/45	Possibly active
10040	15	10/10	1/10 to 1/10	86/45	Inactive
10751	10	10/10	3/10 to 1/10	42/45	Possibly active
10983	20	10/10	5/10 to 1/10	13/45	Slightly active
11223	7·5	10/10	1/10 to 1/10	24/45	Inactive
11255/D	20	10/10	3/10 to 1/10	15/45	Possibly active

It occasionally happens that the average computation of a group is unduly influenced or affected by a single abnormally high count—in such a case the computation of the one mouse is replaced by a figure equal to *double* that of the average computation of the untreated group or if it occurs in the untreated by double the next highest computation in that group. Such adjustment is only occasionally required.

(4) *The use of controls*

The untreated controls are used to assess the intensity of the infection, and additional evidence is often obtainable by consideration of the level of infection in several of the unsuccessfully treated groups. These may show that the untreated group's infection is not truly representative of the general level of infection; if low, then added significance attaches to groups whose infection is obviously reduced, but, if high, then less significance is attached to those groups whose infection appears reduced. In interpreting any such biological test experience must be used and figures not always too rigorously followed. A group of successfully treated 'controls' is also kept and for this purpose tetrachlorethylene has been used at 0·05 c.c. per 20 g. mouse. By the use of this control group one sees the response of the infection to a standard treatment week after week. Since the work started more active compounds have been used as a control treatment, but Table 4 shows the summarized response of numerous groups of mice treated with tetrachlorethylene at two different dosage rates. Two series of summarized results are available for tetrachlorethylene at 0·05 c.c./20 g. (= 2·5 c.c./kg.).

Table 4. *The activity of tetrachlorethylene against* Hymenolepis nana *in mice*

Compound	Dosage rate per 20 g. mouse (c.c.)	No. of mice surviving	Treated/untreated ratios		
			Mice negative	Av. computations	Interpretation
Tetrachlorethylene	0·03	43/50	22/43 to 9/49	32/93	Slightly active
Tetrachlorethylene (1st series)	0·05	139/196	73/139 to 31/253	17/78	Active
Tetrachlorethylene (2nd series)	0·05	34/48	25/34 to 7/50	21/77	Active

Table 5. *An example of the use of 'computation' in the recognition of taeniacidal activity*

Taeniacidal test 42.

Compound	Dosage per 20 g. mouse	Mice negative	Average computations	Total worms	Worm distribution (sizes and numbers)				
					Very large	Large	Medium	Small	Very small
Untreated	—	0/10	199	454	42	54	122	31	*c.* 305
Control treatment	0·02 c.c.	5/10	7	21	0	4	4	11	2
Ext. filicis (male fern)	10 mg.	6/10	8	59	0	0	12	39	8
Ext. filicis	20 mg.	6/7	1	3	0	0	1	2	0

The removal of the larger worms is very obvious in this test and is typical of the tendency of large worms to be removed more readily than small.

V. THE RESPONSE OF THE TEST ORGANISM TO KNOWN TAENIACIDES

The data that exist as to the efficacy of modes of treatment for tapeworms in man are rather indefinite. Success is often recorded when one worm is removed, but there may be more than one worm present. We have tested a number of compounds on both artificially infected dogs and mice. In Table 6 the dosage rates are given per kg. live weight and the usual doses for man are expressed in the same way. In parenthesis the approximate success attained with each dosage rate is also

Table 6. *A comparison of the effect of active compounds on the tapeworms of different hosts*

Compound	Mouse	Man	Dog
Mepacrine	0·4 g./kg. (75 %)	0·02 g. (17–60 %)*	0·05 g. (1/3 cured)
2,2-dihydroxy-dichloro-diphenyl methane	1 g. (100 %)	—	0·1 g. (0/2 cured)
	0·4 g. (20–50 %)		0·2 g. (5/8 cured)
Male fern	1 g. (50–70 %)	0·05 g. (40–80 %)†	0·25 g. (1, or poss. 2, of 3 cured)
			0·5 g. (1 dog unsuccessfully treated —vomited)

* The efficiency of Mepacrine in man was estimated by Halawani (1948) in Cairo to be about 60 %—mostly against *T. saginata*, but Pipkin & Risk (1948) cured only 17–34 % of Lebanese children.

† Reference Mazzotti.

recorded. The most prevalent human tapeworm (*Taenia saginata*, derived from beef) has no hooks for attachment to the gut, whereas those of the dog and *Hymenolepis nana* in mice possess hooks. It is believed that man may be more easily freed from his tapeworm than the dog to which relatively larger doses can be given than would be prescribed for man. The fact that man usually harbours only one tapeworm, whereas dogs may have many may also affect the issue.

The compounds compared in Table 6 all possess taeniacidal or a destructive type of activity. The taeniafugal (expelling) activity of Arecoline is not so well shown on the mouse tapeworm test and is not always detectable.

VI. THE REQUIREMENTS OF A GOOD ANTHELMINTIC FOR TAPEWORMS

The first requirement is, of course, that the treatment be safe and harmless under ordinary conditions of use. It should probably, therefore, be poorly absorbed by the host with specific lethal affinity for the parasite. Compounds which are paralysant but not lethal to the tapeworm (Taeniafuges) may require a purge to assist removal of the tapeworms whilst they are under the influence of the drug. This type of treatment, as with Arecoline in the dog, yields spectacular though inconstant results, as tapeworms may be passed within 20 min. of the treatment. An enema is sometimes necessary to remove tapeworms which have been displaced from the small intestine into the large intestine. It appears that if not removed from the large intestine the tapeworms may re-ascend into the small intestine when they recover from their temporary paralysis.

Modes of treatment which are lethal (Taeniacides) and destructive should be so effective that one can depend on their activity as no evidence of effect will be available if the worms are destroyed and digested. It would be an advantage if the scolex (head) was specifically acted upon as well as the body as regeneration occurs from the tiny head in about 6 weeks in dogs and 3–4 months in man.

Abundant evidence has been obtained in our experimental work that large tapeworms are more readily destroyed than small, and it appears that removal of the large worms permits growth of others whose growth may have been inhibited by the presence of the large worms. It is this easier removal of the large tapeworms which forms the basis of the method of computation in the detection of taeniacidal activity. It can especially be seen when subeffective doses of completely effective compounds are used (Table 5).

A corollary of this different effect of modes of treatment on worms of different sizes may be that young infections are less easy to remove than old. This is possibly an effect of size, and may be due to vitality or to host resistance.

Some taeniacides act slowly, as for example, Mepacrine, with which full effects may not be complete for several days.

Certain taeniacides (possibly male fern) may exert in large doses a taeniafugal effect, the worms being passed alive though probably fatally affected. All of the new compounds found to possess marked activity against tapeworms appear to conform to the taeniacidal type of activity rather than taenifugal.

VII. DISCUSSION

The test described above is simple to operate and using the methods of interpretation described is not difficult to interpret. It can be used with convenience as a routine sorting test for the detection of anthelmintics against tapeworms.

A number of compounds of new types for taeniacides have been found with high activity and these compounds have all shown activity in dogs artificially infected with *Taenia pisiformis*, *T. serialis* and *T. hydatigena*. It is reasonable to expect that they may be active also in man. In addition to its use as a routine sorting test it can be used to study the optimum presentation of taeniacides in relation to the vehicle to be used and the routine of dosing, and also to investigate their mode of action.

By the use of such a test it is expected that the investigation of new anthelmintics and their formulation in an optimum manner may become rational.

SUMMARY

1. A controlled taeniacidal technique is described using mice artifically infected with *Hymenolepis nana* var. *fraterna*.

2. The method of interpretation is based upon a form of computation relating to the number and sizes of worms left in the treated animals as well as the proportion of mice rendered negative by the treatment as compared with untreated controls.

I am pleased to acknowledge my gratitude to Miss M. Okell for her able technical assistance in the development and operation of this testing technique.

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ANTHELMINTIC STUDIES: IV. THE LOSS OF EFFICIENCY BY DIVISION OF THE DOSE

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It has long been customary to use a single large dose in the treatment of worms in animals, but in man there has been a tendency to use a course of doses, especially in the treatment of threadworm. In the case of phenothiazine when used against threadworm the division of the dose has probably been undertaken in order to avoid toxicity. There is, however, no definite evidence that the toxic hazard is reduced in this manner, in fact, it may well be increased as with poorly absorbed substances greater absorption is obtained with the presentation of the treatment over a longer period. This can be clearly demonstrated in laboratory tests.

In the case of gentian violet it is its lack of effect as a single dose anthelmintic that compels its use in a prolonged course of treatment, at doses as high as can be tolerated by the unfortunate patient. Since a suitable laboratory technique has been developed using small animals (Steward, 1955) it has been possible to study the optimal methods of anthelmintic treatment and it can be demonstrated that the division of an effective single dose into smaller parts causes almost complete loss of anthelmintic activity (Tables 1 and 2).

Note on interpretation. Indirect activity is based on a comparison of the number of worms in the treated as compared with an untreated group and is calculated on the following formula:

$$\% \text{ indirect activity} = \frac{\left(\begin{matrix}\text{average count of worms} \\ \text{in untreated group}\end{matrix} - \begin{matrix}\text{average count of worms} \\ \text{in treated group}\end{matrix}\right) \times 100}{\text{average count of worms in untreated group}}.$$

Direct activity is based on a comparison of the worms passed in the faeces with those left in the gut of the treated animals. It is calculated on the following formula:

$$\% \text{ direct activity} = \frac{\text{worms expelled} \times 100}{\text{expelled} + \text{surviving worms}}.$$

It can be seen from Table 1 that division of an effective dose greatly reduces its effect.

Where the total of the divided dosage is 1½ times the single dose the effect does not appear to be reduced if it is given in three doses at one dose per day for 3 days; it appears a little reduced where 1½ times the single dose is given as five doses spread over 2½ days. At 1 dose per day for 5 days similar dosage has appreciably less effect (Table 2).

With another anthelmintic the loss of activity was still more striking when given at 1½ times the total dose divided into five doses, given twice daily.

It appears that a suitable concentration of anthelmintic requires to be attained over a short period to attain optimum effect; a lower concentration over a more extended period is less effective.

CONCLUSION

A single large dose of certain anthelmintics is more effective than when given in divided doses.

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(*MS. received for publication* 13. III. 1954.—Ed.)

Table 1. *The reduced effect of divided doses of an anthelmintic effective as a single dose*

Treatment	Dosage routine per 100 g. rat	No. of rats	Average worm count		Individual worm counts	Percentage anthelmintic effect (indirect)
			Worms in gut	Worms excreted in faeces		
Test 189						
Untreated	—	10	302	1	Gut: 340, 336, 320, 260, 340, 264, 210, 196, 390, 368 Faeces: 2, 1, 0, 0, 0, 1, 0, 1, 1, 0	—
Phenothiazine	100 mg. as one dose	8	11	118	Gut: 28, 0, 6, 0, 0, 23, 12, 15 Faeces: 196, 70, 212, 24, 59, 94, 120, 166	96
Phenothiazine	100 mg. as 9 doses (11 mg. given over 4½ days at 2 doses a day)	16	196	21	Gut: 136, 110, 300, 276, 114, 164, 120, 210, 286, 220, 250, 80, 212, 196, 264, 190 Faeces: 42, 15, 13, 21, 0, 53, 14, 50, 16, 14, 0, 0, 62, 26, 4, 5	35
Test 193						
Untreated	—	10	72	0	Gut: 102, 77, 52, 91, 36, 85, 30, 106, 82, 58 Faeces: 1, 0, 0, 0, 0, 0, 0, 0, 0, 1	—
Phenothiazine	100 mg. as one dose	8	34	36	Gut: 0, 18, 0, 5, 44, 2, 76, 128 Faeces: 12, 49, 55, 53, 18, 1, 22, 70	53
Phenothiazine	100 mg. as 9 doses (11 mg. given over 4½ days at 2 doses a day)	10	88	5	Gut: 56, 56, 130, 90, 86, 64, 98, 68, 126, 102 Faeces: 0, 3, 2, 1, 0, 2, 32, 0, 8, 0	0

Table 2. *Single anthelmintic dose compared with different regimes of divided doses equal in total to 1½ times the single dose*

Treatment	Dosage routine per 100 g. rat	No. of rats surviving	Average worm count: Worms in gut	Average worm count: Worms expelled in faeces	Individual worm counts	Percentage anthelmintic effect: Indirect	Percentage anthelmintic effect: Direct
Test 195							
Untreated	—	10	95	1	Gut: 76, 93, 20, 102, 146, 86, 140, 74, 60, 148 Faeces: 0, 3, 0, 3, 1, 0, 0, 0, 0, 0	—	—
Phenothiazine	75 mg. in 1 dose	15/15	17	29	Gut: 17, 50, 1, 36, 26, 17, 0, 10, 32, 5, 5, 3, 3, 18, 36 Faeces: 38, 2, 34, 2, 68, 30, 6, 38, 49, 5, 17, 10, 69, 0, 67	82	63
Phenothiazine	110 mg. in 3 doses at one dose per day. (=37 mg. × 3 = 1½ × control dose)	14/15	16	24	Gut: 11, 15, 1, 13, 45, 13, 7, 8, 21, 29, 12, 27, 6, 12 Faeces: 34, 7, 32, 22, 15, 16, 10, 20, 18, 30, 59, 10, 30, 47	83	60
Phenothiazine	110 mg. in 5 doses at 1 dose per day (=22 mg. × 5 or = 1½ × control dose)	15/15	41	11	Gut: 14, 45, 36, 6, 74, 28, 56, 80, 26, 36, 98, 8, 13, 58, 36 Faeces: 15, 0, 3, 14, 7, 0, 48, 26, 28, 2, 0, 0, 2, 11, 16	57	21
Phenothiazine	110 mg. in 5 doses at 2 doses per day (=22 mg. × 5 in 2½ days = 1½ × control dose)	13/15	25	20	Gut: 5, 38, 10, 26, 16, 56, 53, 29, 56, 9, 13, 4, 10 Faeces: 53, 69, 7, 33, 30, 5, 18, 7, 11, 5, 1, 4, 23	73	44

THE MANGE-CAUSING MITES OF THE GENUS *PSORERGATES* (ACARINA: MYOBIIDAE) WITH DESCRIPTION OF A NEW SPECIES FROM A SOUTH AFRICAN MONKEY

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Department of Entomology, South African Institute for Medical Research, Johannesburg

(With 7 Figures in the Text)

A male specimen of the monkey, *Cercopithecus aethiops pygerythrus* (Cuvier), caught near Bedford, Eastern Cape, was sent to us by Dr P. A. D. Winter of the Polio Research Foundation, Rietfontein. On arrival at Rietfontein this monkey showed slight symptoms of a mange, described by Dr Winter as follows: 'The lesion was scaly with underlying patches of erythema. Over the lesion the hair was thin. On scraping, much of the scaly material could be taken away, revealing irregular, raised erythematous patches. These patches were distributed particularly on the dorsal aspects of the limbs and there were also several of them on the chest, abdomen and head.

'The monkey was treated with an ointment containing mercurochrome, acriflavine and sulphonamide. After about two weeks there seemed to be some response to the treatment in that there was less scaliness.'

Aware of the possibility that this mange might be due to an interesting and hitherto undescribed species of mite, we kept the monkey for several months hoping that the treatment had not been too effective and that the mites would increase again. During this period there was little change in the symptoms. The hair began to grow again in the thinned areas, but the skin remained scaly. Finally it was decided to kill the monkey and to examine the skin for the presence of mites.

Pieces of skin from several parts of the body, especially the head and limbs, were dissolved in a weak solution of potassium hydroxide. Examination of these samples revealed the presence of vast numbers of mites.

These mites represent a new species belonging to the genus *Psorergates* (Myobiidae), founded by Tyrrell in 1883 to include *P. simplex* Tyrrell, a species first recorded from the ear of the house mouse, *Mus musculus* L. *P. simplex*, originally described from Canada, was subsequently recorded by Oudemans (1897, and in litt.) and Vitzthum (1929) from Europe, and by Lavoipierre (1946) from South Africa where it was found to cause a severe and fatally ending mange in stocks of *Rattus* (*Mastomys*) *natalensis* (Smith).

The second species of *Psorergates* was described by Michael in 1889 from the mouse, *Arvicola agrestis* L., in Great Britain. He saw only the male, on which he based the new genus and species *Goniomerus musculinus*. This species was later mentioned by Neumann (1893) and by Vitzthum (1929), but most probably no further specimens were collected.

The last species, *Psorergates ovis*, was described by Womersley (1941) from Australian sheep, in which it produces 'a chronic irritation of the skin, mainly along the sides and flanks, although specimens have been recovered from most regions of the body. The appearance of the fleece is similar to that of infestation by the common biting louse (*Bovicola ovis*).'*

Dr H. Womersley was kind enough to send us several female specimens of *Psorergates ovis* for comparison with our new species. The specimens of *P. simplex* depicted in this paper were taken from laboratory stocks of *Rattus natalensis* in South Africa. *Psorergates musculinus* has remained unknown to us and as both the original description and drawing are inadequate by present-day standards, we have not been able to include this species in our key.

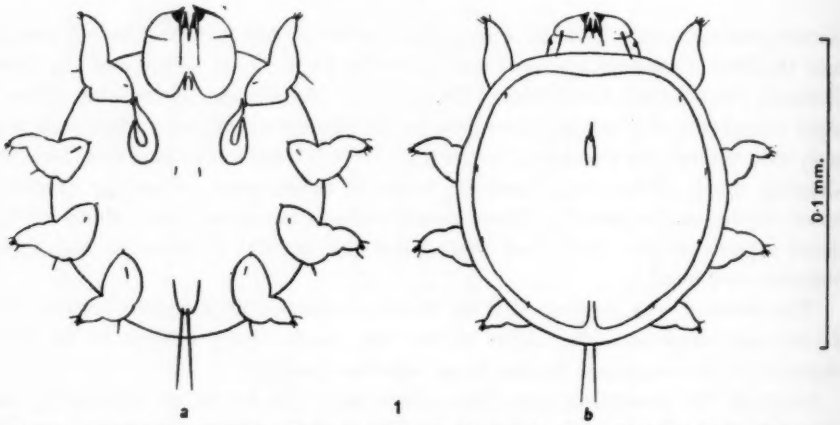

Fig. 1. *Psorergates cercopitheci* n.sp. (*a*) Ventral view of male; (*b*) dorsal view of male.

KEY TO THE *PSORERGATES* SPECIES†

1 (2). Dorsal shield with inconspicuous, short, fine hairs. 3

2 (1). Dorsal shield with long (± 18 μ), stiff hairs (Figs. 4, 5). Body length: ♂ 167 μ, ♀ 189 μ; width: ♂ 116 μ, ♀ 162 μ. Australia, from sheep. **ovis** Womersley, 1941

3 (4). First pair of apodemes thick, loop-shaped, apodemes 2–4 indistinct; long (± 15 μ) dorsal seta on palp. In ♂, penis short, situated at level of 2nd pair of legs; one pair of terminal hairs arising from single median tubercle at posterior tip of body (Figs. 1, 2). Body length (including mouthparts): ♂ 105–125 μ, ♀ 120–132 μ; width: ♂ 90–107, ♀ 100–115. South Africa, from *Cercopithecus aethiops pygerythrus* (Cuvier). **cercopitheci** n.sp.

4 (3). First pair of apodemes slender with terminal hook, other three pairs distinct; short, inconspicuous dorsal seta on palp. In ♂, penis long, extending from level of 4th to 1st pair of legs; terminal hairs and median tubercle absent (Figs. 6, 7). Body length (including mouthparts): ♂ 128–134 μ, ♀ 146–150 μ; width: ♂ 108–112 μ, ♀ 122 μ. Described from *Mus musculinus* Linn., N. America, recorded by Lavoipierre—from *Rattus natalensis* (Smith), South Africa. **simplex** Tyrrell, 1883

* Since going to press, O. G. H. Fiedler & R. du Toit (*J. S. Afr. Vet. Med. Ass.* **25** (2), 21, 1954) have reported the occurrence on sheep in South Africa, of mites said to be closely related to *P. ovis*. The authors have now confirmed (personal communication) that these mites are identical with *P. ovis*.

† According to the drawing given by Michael (1889) *P. musculinus* is relatively more slender than the species given in the above key, its length (115 μ) being 1·5 times its breadth (105 μ), and the femora have more pronounced posterior angles. (Fig. 3).

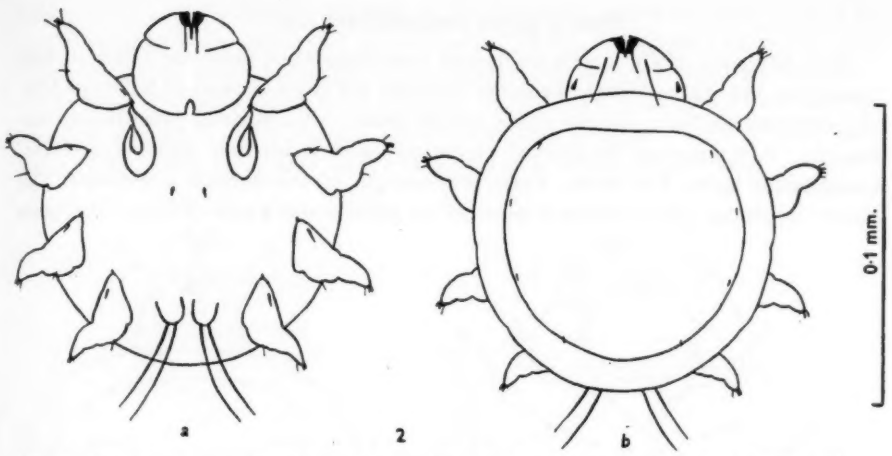

Fig. 2. *Psorergates cercopitheci* n.sp. (*a*) Ventral view of female; (*b*) dorsal view of female.

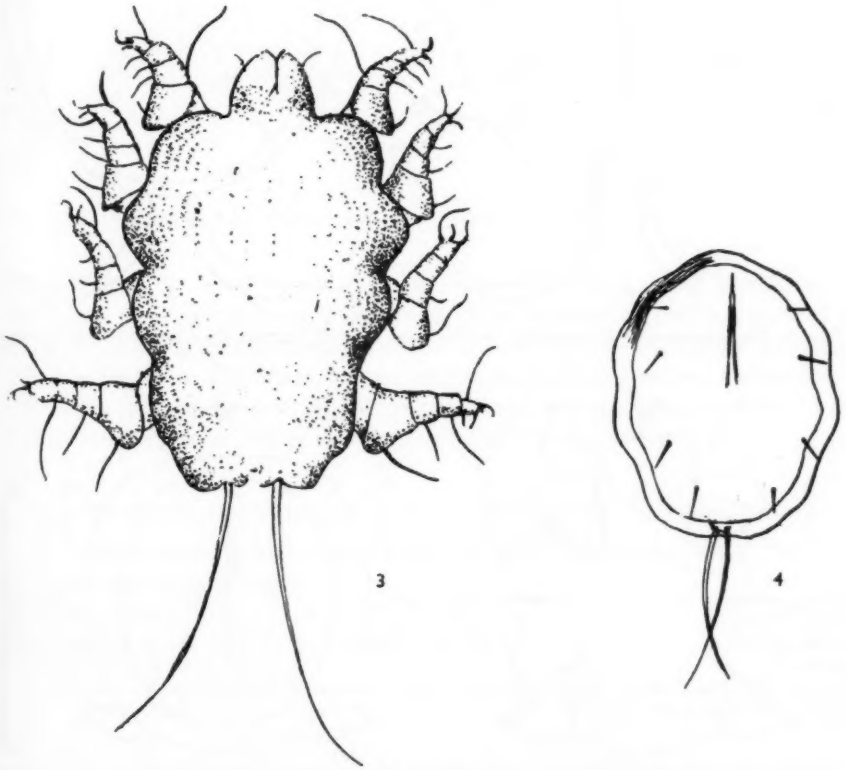

Fig. 3. *Psorergates musculinus* Michael. Dorsal view of male (after Michael, 1889).
Fig. 4. *Psorergates ovis* Womersley. Dorsal view of male (after Womersley, 1941).

Psorergates cercopitheci n.sp.

Male (Fig. 1). Body length (including mouthparts) of holotype 110 μ, of four paratypes 105–125 μ. Width (between 2nd and 3rd pairs of legs) of holotype 90 μ, of paratypes 90–107 μ. Dorsal shield almost round, with slightly concave anterior margin. It appears to be divided posteriorly and bears four pairs of short inconspicuous hairs. The outer, uncovered margin of the dorsum is striated. The venter bears one pair of delicate setae in the middle and a pair of long (50 μ) hairs

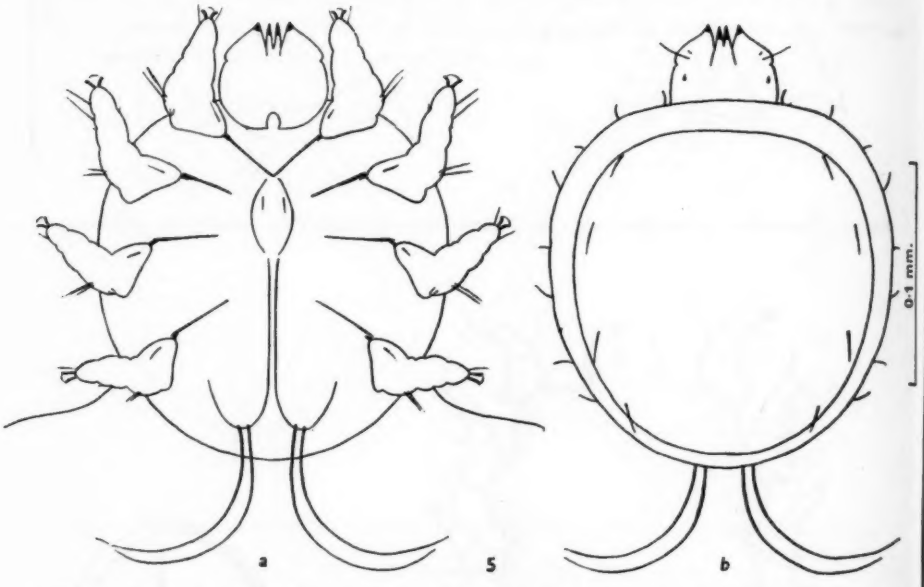

Fig. 5. *Psorergates ovis* Womersley. (*a*) Ventral view of female; (*b*) dorsal view of female.

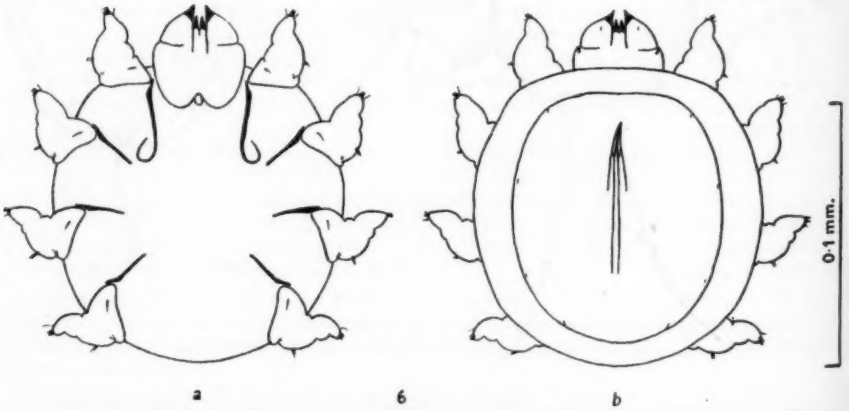

Fig. 6. *Psorergates simplex* Tyrrell. (*a*) Ventral view of male; (*b*) dorsal view of male.

arising from a single median tubercle at the posterior end of the body. The first pair of apodemes are thick and loop-shaped. The remaining three pairs are very faintly indicated in the holotype, but indistinguishable in the other specimens.

The penis is situated dorsally at the level of the second pair of legs and has the structure indicated in the figure. Each palp bears dorsally a long ($\pm 15\mu$) seta and a short, blunt outer seta, and terminates in a well-chitinized claw.

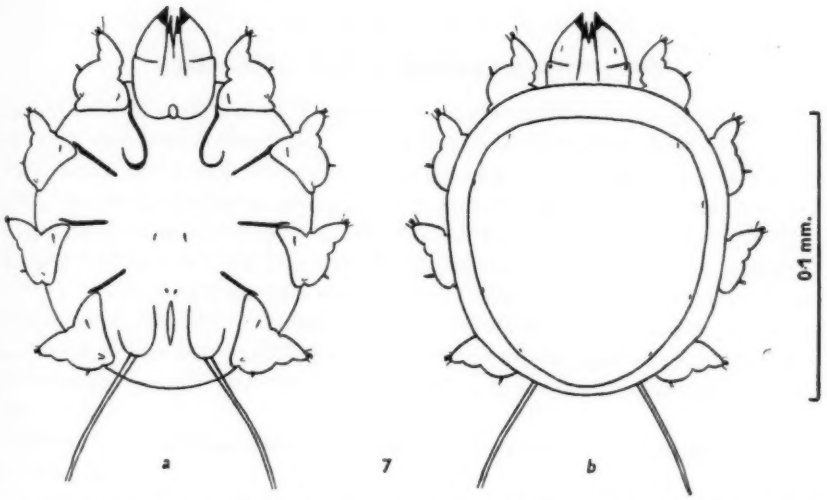

Fig. 7. *Psorergates simplex* Tyrrell. (*a*) Ventral view of female; (*b*) dorsal view of female.

Female (Fig. 2). Body length of ten specimens 120–132μ, width 100–115μ. It differs from the male in having the dorsal shield without a posterior slit and in having two pairs of long terminal hairs arising from two postero-ventral tubercles.

The species is based on five males and ten females from the monkey, *Cercopithecus aethiops pygerythrus* (Cuvier), Cape Province, South Africa, 20 August 1953. There are, however, numerous other specimens, mainly females, before us.

SYSTEMATIC—SYNONYMIC LIST OF THE GENUS *PSORERGATES*

Psorergates Tyrrell, 1883; Vitzthum, 1929; Womersley, 1941

Syn. *Goniomerus* Michael, 1889

1. *cercopitheci*, n.sp. — S. Africa
2. *musculinus* (Michael, 1889; Vitzthum, 1929). — Great Britain
3. *ovis* (Womersley, 1941). — Australia
4. *simplex* (Tyrrell, 1883; Neumann, 1893; Oudemans, 1897; Vitzthum, 1929; Lavoipierre, 1946). — Europe, N. America, S. Africa

We wish to express our appreciation to Dr G. L. van Eyndhoven (Amsterdam Museum), Dr. P. A. D. Winter (Polio Research Foundation) and Dr H. Womersley (South Australian Museum) for their helpful co-operation.

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SOME NEW AND LITTLE-KNOWN MITES OF THE GENUS *MYOCOPTES* CLAPARÈDE (ACARINA: LISTROPHORIDAE)*

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(With 16 Figures in the Text)

INTRODUCTION

Claparède (1869) established the genus *Myocoptes* for a mite, found upon a house-mouse (*Mus musculus musculus* L., 1758), which had been described by C. L. Koch (1844) under the name *Sarcoptes musculinus* (syn. *Listrophorus larisi* Vorobiov, 1938). In this genus sexual dimorphism is pronounced; the female has legs iii and iv modified and provided with processes with which it can maintain a firm grasp on the fur of its host. In the male this modification is only present on legs iii.

Poppe (1889), in describing a new species of mite found upon a hamster (*Cricetus cricetus cricetus* L., 1758 = *Cricetus frumentarius* Pallas, 1711), established for it the new genus *Criniscansor with* the new species *C. criceti* Poppe as the genotype. The genus *Criniscansor* Poppe, 1889, is, however, a synonym of *Myocoptes* Claparède, 1869.

Several other workers have since described new species in this genus and these are listed below:

New species	Type host
Myocoptes tenax Michael, 1889	*Arvicola agrestis agrestis* L., 1761
M. glirinus Canestrini, 1895	*Glis glis glis* L., 1766
M. hintoni Hirst, 1919	*Sciurus vulgaris vulgaris* L., 1758
M. ramboutsi Eyndhoven, 1946	*Mus musculus* L. *forma brunnea* (?)
M. kalrai Radford, 1947	*Gerbillus* sp.

No figure was given by Hirst (1919) for his species *M. hintoni* of which he possessed only the female. The author has been able to obtain females of this species which are here re-described and figured. The male is unknown.

Eyndhoven (1946) has described but not figured his species, *M. ramboutsi*.

Over a number of years the author has received from various sources a number of specimens, amongst which are some which do not conform to the descriptions of the species already known. These species are herein described and figured; the drawings have been made by the author from specimens in his private collection. Figs. 15 and 16 are enlarged from the tracings of the figures of *Criniscansor criceti* given by Poppe (1889).

The author wishes to thank Dr R. F. Lawrence, Natal Museum, Pietermaritzburg, South Africa, for his observations on the new species; Dr E. W. Jameson, jun., Assistant Professor of Zoology, University of California, Davis, California, U.S.A., for providing the specimens *japonensis* and *jamesoni* which I name in recognition of his contributions to Acarology.

* This work has been made possible by a grant from the Nuffield Foundation, London.

To the collectors named later in this paper I wish to tender my thanks for their donations of specimens.

Family LISTROPHORIDAE Canestrini, 1892
Subfamily MYOCOPTINAE Gunther, 1942
Genus MYOCOPTES Claparède, 1869

Myocoptes japonensis n.sp.

The male venter (Fig. 1) shows the body to be mainly elliptical. Legs i and ii are placed on the anterior third of the body and each bears a tarsal sucker. Legs iii are placed on the lateral edge of the body about midway between the anterior and posterior end of the body; the second segment is broader and longer than the others and has a grooved surface; the third segment has a stout spur on its ventral surface; the fourth segment has a peg-like spur. Legs iv are placed on the posterior third of the body close to the lateral edge. Leg iv is devoid of any setae except the stiff seta on the distal, lateral edge; the tarsus also bears a short, stout spur or claw.

Chaetotaxy. A long stout seta is borne on the posterior margin of coxa i; a similar seta is borne on the anterior edge of coxa ii; a third seta is borne upon a peduncle flanking leg iii internally; two setae lie between the lateral edge of the body and the genital pore. On each side of the posterior end of the body there are three stout bristles, the median bristle being longer and stouter than the others. The posterior end of the body is prolonged into two triangular hyaline processes, each bearing a single seta.

The soft integument between coxae ii and iii is provided with scale-like processes similar to those in *Sarcoptes scabiei* (Degeer, 1778). Between coxae iv is the genital pore; in the type specimen the curved penis is directed posteriorly and is bifid apically. A pair of fine setae is placed between the genital pore and the genital suckers; the latter being flanked by chitinized platelets.

The dorsum of the male is provided with transverse striations across the centre and with striations down the sides, leaving the posterior half of the dorsum devoid of striations (Fig. 2). The capitulum bears a pair of short setae anteriorly, with a pair of longer and stouter setae at its base which extend almost to the V-like mark between the scapular setae. The external scapular setae are stout and long and extend almost to lateral setae ii. The internal scapular setae are considerably shorter than the externals. Chaetotaxy of the posterior tip of the dorsum as in the figure.

Between legs iv there is a chitinized process shaped like a tuning fork, on which there is a pair of long, stout setae which are almost equal in length to the third pair of laterals. On the lateral edge of the body between legs ii and iii are two long, stout setae, the posterior being the shorter. Known only from the type specimen.

Female. Unknown.
Host. A mouse (*Clethrionomys rufocanus smithii* Thomas, 1905).
Locality. O'hara, Honshu, Japan. Coll. Dr E. W. Jameson, jun., 30 March 1952.
Measurements. ♂, 0·255 × 0·15 mm.

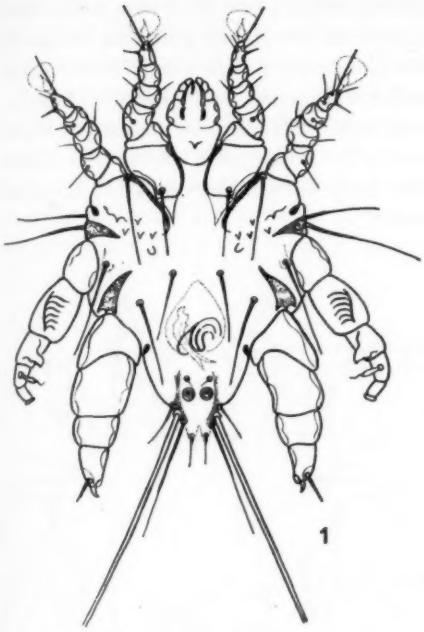

Fig. 1. *Myocoptes japonensis* n.sp. Male venter.

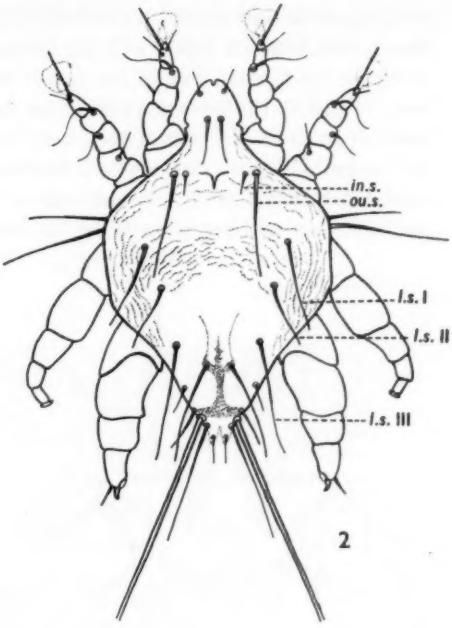

Fig. 2. *M. japonensis* n.sp. Male dorsum. *in.s.*, inner scapular setae; *ou.s.*, outer scapular setae; *ls.* I, II, III, lateral setae.

Myocoptes jamesoni n.sp.

The female venter (Fig. 3) shows the body to be elliptical, all legs borne on the anterior half of the body. Epimera all separate. Coxa i bears a seta on the posterior edge; coxa iii with one seta on the antero-lateral edge and another seta on the posterior edge. Lying on a level with the posterior edge of coxa iii and flanking the genital pore is a pair of long setae. Slightly anterior to coxa iv and posterior to the pair of genital setae is a pair of long setae. Posterior to the genital pore near the median line of the venter is a pair of very short setae, flanked on each side by a line of pointed, scale-like processes. Between the genital pore and the anus the venter is ornamented with rows of these scale-like processes. Anterior to the anus is a pair of setae whilst a second pair of setae flank the anus. Laterally, level with the anus is a series of semicircular striations. The posterior end of the body bears a pair of long, stout terminal bristles.

Legs i and ii are placed on the anterior third of the body; each leg is provided with a few setae and a tarsal sucker. Leg iii has a seta on the trochanter; a corrugated process occupies the central position on the femur; the genu bears the spur characteristic of the genus; the tibia and tarsus each bear a stout spur as in the figure. Leg iv similar to leg iii.

The female dorsum (Fig. 4) has three pairs of setae on the capitulum, the postero-median setae being the longest. Level with legs ii is a pair of scapular setae which

are long, stout and extend posteriorly to lateral setae i. Lateral setae i level with a line drawn between legs ii and iii; lateral setae iii are placed posterior to legs iv, closer to the median line of the body; lateral setae iv nearer the posterior tip of body. Close to the posterior end of the body are the long, stout, terminal bristles; anterior to these is a pair of very long, stout setae. Striations are apparent round the lateral and posterior edges of the dorsum, the whole area between the dorsal setae being nude. On the lateral edge of the body between legs ii and iii there are two setae, the anterior seta being the shorter.

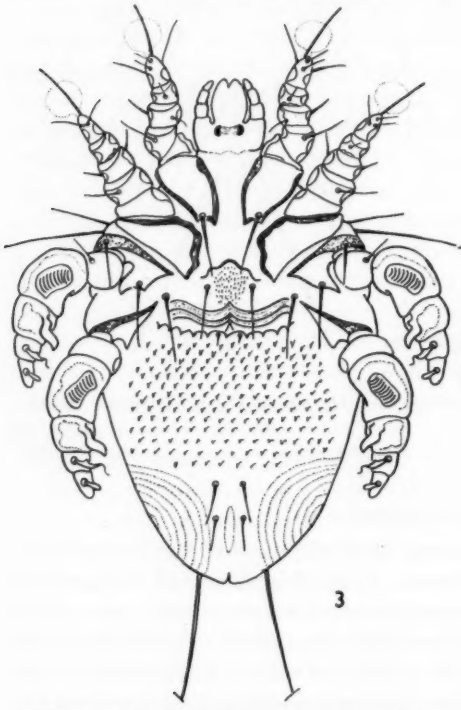

Fig. 3. *M. jamesoni* n.sp. Female venter.

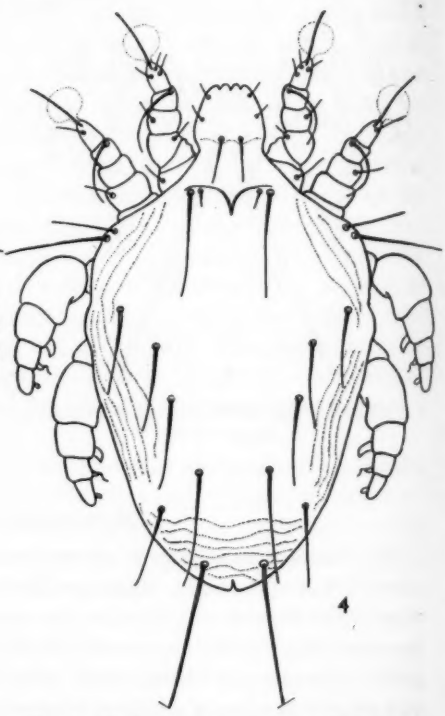

Fig. 4. *M. jamesoni* n.sp. Female dorsum.

Male. Unknown.
Host. A mouse (*Microtus pennsylvanicus pennsylvanicus* Ord. 1815).
Locality. Ithaca, New York, U.S.A. Coll. Ed. Riley, 24 October 1949.
Measurements. ♀, 0·180 × 0·105 mm.

Myocoptes canadensis n.sp.

The male venter (Fig. 5) shows legs i and ii to be borne on the anterior part of the body; legs iii placed laterally, midway between the anterior and posterior ends of the body; legs iv midway between the third pair of legs and the posterior end of the body. Legs i and ii short, subsimilar, with few setae and bearing a tarsal sucker; leg iii stouter and with characteristic armature on the genu, tibia and tarsus.

Leg iv stout, apparently of four segments, the femur and genu being fused together; the tarsus provided at its distal end with a strongly chitinized, pronged process; on the outer flank of the tarsus is a stout seta.

Coxae all separate; coxa i and ii each provided with one seta; coxa iii with two setae, one anteriorly the other on the posterior projection. Between coxae ii is a pair of long setae, with a second pair of setae between the pore and legs iv. Posterior to the pore there is a pair of very short setae. Genital suckers placed well back near the posterior tip of body, surrounded by two strongly chitinized platelets, between the anterior tips of which is a pair of small setae.

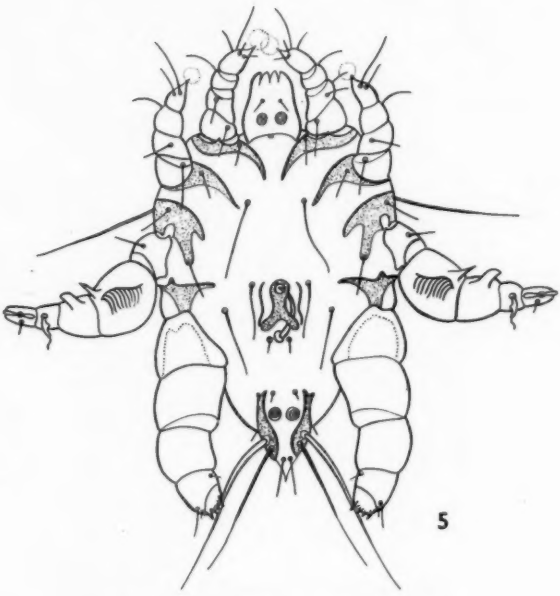

Fig. 5. *M. canadensis* n.sp. Male venter.

The posterior tip of the venter is provided with three pairs of setae; a pair of very small setae flanking the terminal bristles; on the internal side of the terminal bristles is a pair of long, slender setae; the third pair of setae are borne on the triangular hyaline flap which terminates the body. Penis long, stout, curved, with a strongly chitinized process as figured.

The male dorsum (Fig. 6) shows the capitulum to possess one pair of setae on the antero-lateral edge; posterior to the capitulum is a pair of setae, extending to the scapular setae. Scapular setae level with lateral setae i; long, stout and reaching laterals ii. Inner scapular setae longer than in other species. Laterally, between legs ii and iii are the customary two pairs of setae, the anterior being the shorter. Lateral setae i level with legs iii; lateral setae ii between legs iii and iv; lateral setae iii level with legs iii; lateral setae iv close to the postero-lateral edge of body. A long, slender, chitinized, fork-like process on the posterior third of the body

carries a pair of long stout setae. On the terminal, hyaline, triangular tip of body is a pair of small setae.

Female. Unknown.

Host. A lemming (*Dicrostomys* sp.).

Locality. Moosonee, Ontario, Canada. Coll. W. Whitehead, 11 September 1935.

Measurements. ♂, 0·240 × 0·165 mm.

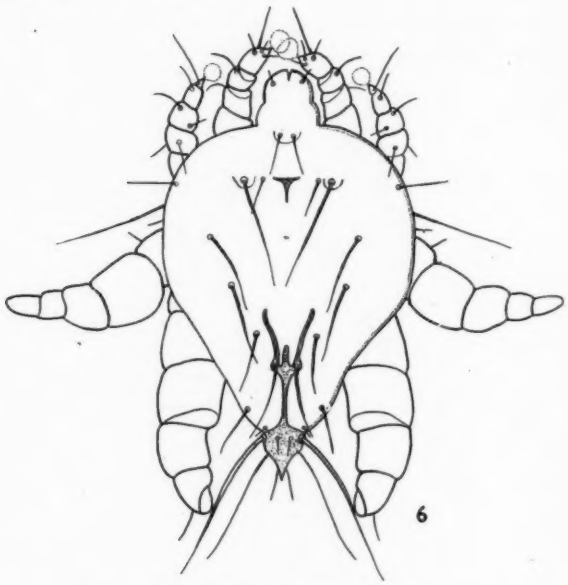

Fig. 6. *M. canadensis* n.sp. Male dorsum.

Myocoptes hintoni Hirst, 1919

The female venter (Fig. 7) shows epimera i to converge towards the centre but without forming a sternum. Epimera ii and iii close to the lateral edge of the body but separated from each other. On the capitulum there is a pair of setae and a chitinized anvil-like plate. One seta on coxae i and ii. Between epimera ii is a pair of long setae which extend to the genital pore. Close to coxa iii and level with anterior of genital pore is a seta on each side. Two pairs of long, slender setae flank the genital pore, with a third pair of setae posterior to the pore. Anterior of the terminal bristles are three pairs of long setae. Venter ornamented with rows of V-shaped scales between the genital pore and the three pairs of posterior setae. Laterally and posteriorly there are striations round the scale-like area.

The possession of two pairs of terminal bristles in the female is the feature which distinguishes this species from all others in the genus. Legs i and ii short, stout and subequal, each with a tarsal sucker; legs iii and iv with armature as in the figure.

The female dorsum (Fig. 8) shows the long external scapular setae level with legs ii; lateral setae i level with legs iii and two accessory setae anteriorly; lateral

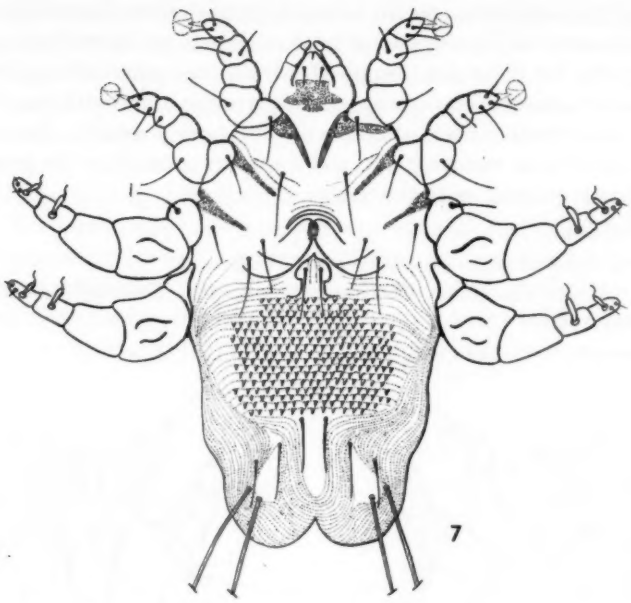

Fig. 7. *M. hintoni* Hirst, 1919. Female venter.

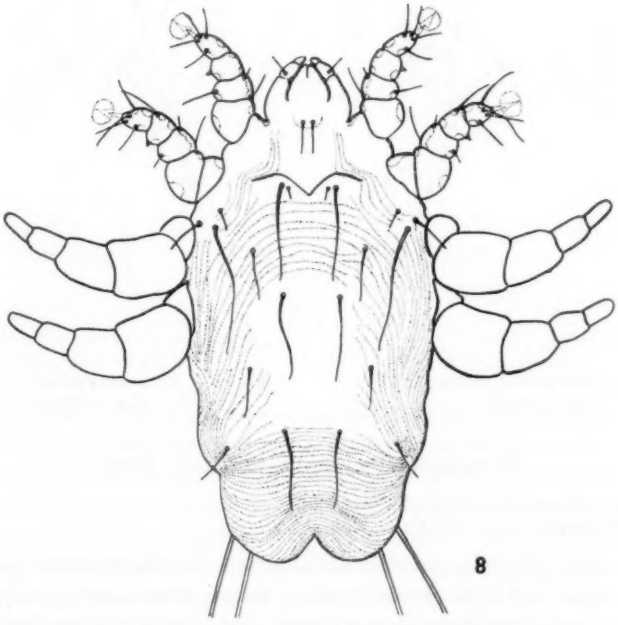

Fig. 8. *M. hintoni* Hirst, 1919. Female dorsum.

setae ii slightly posterior to legs iii; lateral setae iii shorter, placed a short distance beyond the posterior of legs iv; lateral setae iv close to the lateral edge of the body.

In this species the dorsum is also provided with two pairs of long, stout median setae in line with the scapular setae: one pair lying on a line with legs iv, the other pair being placed further back, almost level with lateral setae iv. The central area of the dorsum between median setae i and ii appears to be nude; the remaining area of the dorsum is striated as in the figure.

Male. Unknown.

Host. Red squirrel (*Sciurus vulgaris* L., 1758).

Locality. Hurn Common, Christchurch, Hants, England. Coll. J. Hake, 15 September 1946.

Measurements. ♀, 0·340 × 0·170 mm.

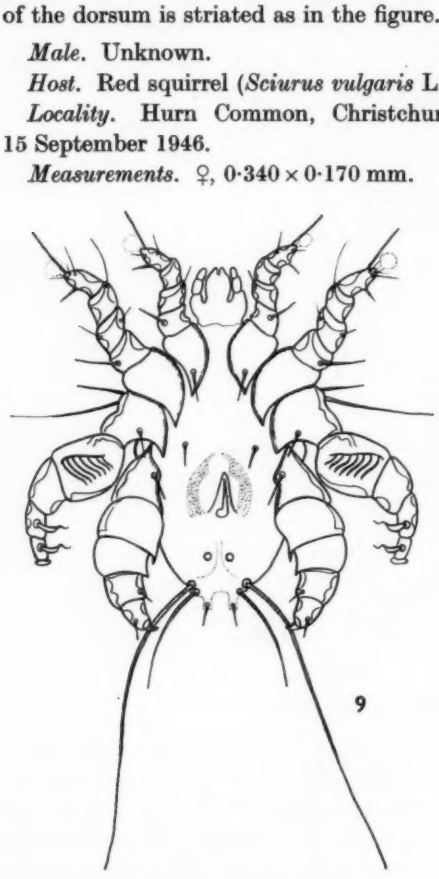

Fig. 9. *M. musculinus* (Koch, 1844). Male venter.

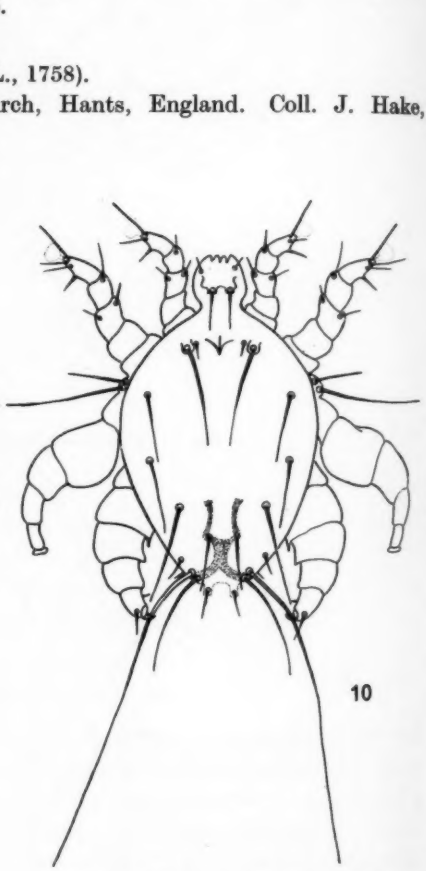

Fig. 10. *M. musculinus* (Koch, 1844). Male dorsum.

Myocoptes musculinus (Koch, 1844)

syn.: *Sarcoptes musculinus* Koch, 1844.
Listrophorus larisi Vorobiov, 1938.

The male venter (Fig. 9) shows coxae i, ii and iii to be placed close together. Legs i shorter than legs ii but both provided with a tarsal sucker; legs iii short, provided with armature as figured; legs iv very broad, particularly the proximal segments; its tarsus provided with a long, curved claw and a stiff seta on the external edge.

Coxae i, iii and iv each with one seta. Anterior to the genital pore is a pair of setae. The posterior tip of the venter has a hyaline wing which is indented medially, each lobe of the wing bearing a seta. Flanking these, laterally, are two pairs of long, stout setae; the outer setae being much longer and stouter than the inner. Genital pore surrounded by an arch-like chitinized plate; penis short and spine-like.

The male dorsum (Fig. 10) shows the scapular setae to be long, extending to lateral setae ii, an arrow-head-like process lies between the scapulars. Lateral setae i on a line between legs ii and iii; lateral setae ii between legs iii and iv; lateral setae iii longer and stouter than laterals i and ii, extending to tarsi of legs iv; lateral setae iv close to the postero-lateral edge of the body. At the median, posterior end of the body is a chitinized, tuning-fork like process which carries a pair of long, stout setae.

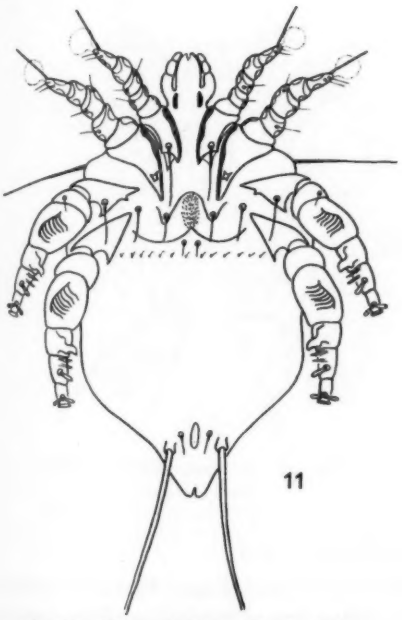

Fig. 11. *M. musculinus* (Koch, 1844). Female venter.

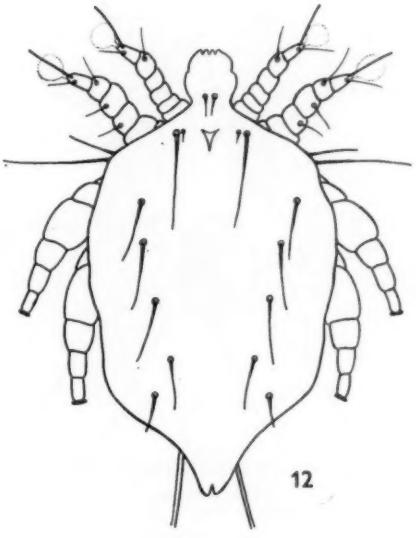

Fig. 12. *M. musculinus* (Koch, 1844). Female dorsum.

The female venter (Fig. 11) shows the posterior end of the body to be greatly reduced. Legs placed well forward on the anterior half of the body. Coxae i close together, each bearing a single, long seta; coxae ii close to coxae i, bearing a strongly chitinized process; coxae iii with a single seta each; coxae iv nude. Anterior to the genital pore is a pair of setae lying between coxae iii and iv; flanking the pore is a pair of setae; posterior to the pore is a pair of very short setae and a line of scale-like processes. A pair of setae flanks the anus.

Legs i and ii with a tarsal sucker and a few setae; legs iii and iv short, stout and with armature as figured.

The female dorsum (Fig. 12) shows the external scapulars to be long, stout and placed close to the antero-lateral edge of the body, level with legs ii; the median, arrow-head process is long and stout. Lateral setae i short, level with legs iii; lateral setae ii lying between legs iii and iv; lateral setae iii lying behind legs iv; lateral setae iv much shorter and close to the postero-lateral edge of the body. A median pair of setae placed between laterals iii and iv. Between legs ii and iii on the lateral edge of the body are two pairs of long, stout setae.

Host. House mouse (*Mus musculus musculus* L., 1758).

Locality. Manchester, Lancashire, England. Coll. C. D. Radford, 15 January 1934.

Measurements. ♂, 0·225 × 0·135 mm.; ♀, 0·320 × 0·150 mm.

This species appears to be cosmopolitan, having been recorded from the house-mouse in many countries. It has also been recorded from laboratory white mice.

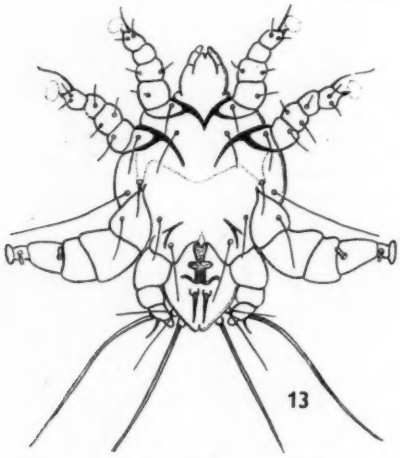

Fig. 13. *M. tenax* Michael, 1889. Male venter.

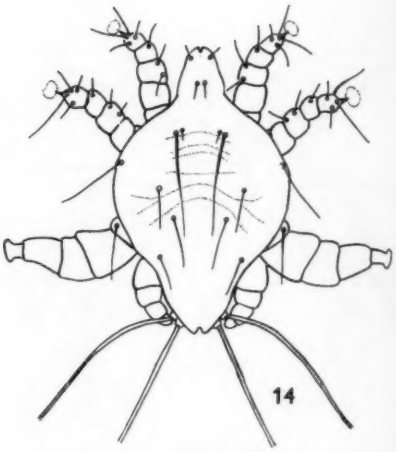

Fig. 14. *M. tenax* Michael, 1889. Male dorsum.

Myocoptes tenax Michael, 1889

The male venter (Fig. 13) shows the characteristic squat shape. Epimera i united medially; epimera ii pointing towards the median line of the body. Legs i and ii short, stout, provided with a tarsal sucker and few setae; legs iii long, stout, provided with a single peg-like spine on the fourth and fifth segments; at the distal end of the fifth segment there is a disk-like recurved flap; legs iv greatly reduced, not extending to the posterior tip of the body. Chaetotaxy of the venter as figured. The genital pore placed posterior to legs iv and surrounded by a heavily chitinized plate. Posterior to the pore there is a pair of short, but very stout setae which are borne upon pedunculated bases. At the postero-lateral edge of the body are two pairs of long, slender setae and the terminal bristles, two on each side, which diverge outwards.

The male dorsum (Fig. 14) shows the external scapular setae to be stout, exceptionally long, extending to legs iii. Lateral seta i close to the median line of the

dorsum, between legs ii and iii; lateral setae ii much longer, closer together and level with legs iii; lateral setae iii nearer the lateral edge of the body, level with legs iv. Posterior to legs ii is a pair of long setae on the lateral edge of the body.

Host. A vole (*Arvicola agrestis agrestis* L., 1761).

Locality. Bridgnorth, Shropshire, England. Coll. Frances Pitt, 17 October 1933.

Measurements. ♂, 0·260 × 0·140 mm.

Myocoptes criceti (Poppe, 1889)

syn.: *Criniscansor criceti* Poppe, 1889.

The male venter (Fig. 15) shows the epimera to be pronounced and widely separated. Legs i and ii on the anterior half of the body at the lateral edge; legs stout, subsimilar; each with a tarsal sucker and few setae. Legs iii long, placed on the postero-lateral part of the body; trochanter and femur stout, fused together, the

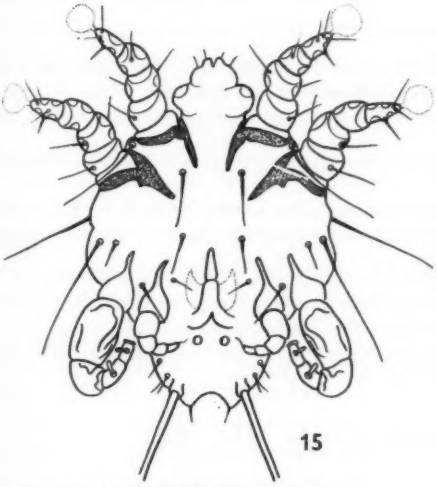

Fig. 15. *M. criceti* (Poppe, 1889). Male venter.

Fig. 16. *M. criceti* (Poppe, 1889). Female venter.

inner surface grooved; genu, tibia and tarsus provided with peg-like spurs. Legs iv greatly reduced in length and width, not reaching the posterior end of the body; situated ventrally between legs iii and the genital pore. Venter provided with several pairs of setae; a pair of long setae on the lateral edge between legs i and ii; a pair of shorter setae between epimera ii, with two pairs near the lateral edge, the outer pair are presumably the lateral setae. Between epimera iii and iv is a pair of setae on each side of the body. Anterior to the terminal bristles are two pairs of short setae, and a pair of shorter setae at the extreme tip.

The genital pore lies between coxae iv, almost in the centre of the posterior half of the venter. The penis is large, stout and conical, flanked on each side by a chitinized flap and two setae; a pair of genital suckers are placed posterior to the pore.

The female venter (Fig. 16) shows legs i and ii to be placed on the lateral edge of the body, well forward towards the capitulum. Legs iii and iv placed on lateral

edge equi-distant between the anterior and posterior ends of the body. Legs ii and iii short, stout and subequal, each with a tarsal sucker; legs iii and iv short, stouter than legs i and ii. Anterior to coxa iii is a short seta and the customary long, stout lateral seta.

The genital pore placed between coxae iii, flanked by three pairs of setae, two pairs anteriorly and one posteriorly.

In Poppe's original description of the species only a brief reference is made to the dorsal surface and the scapular setae. As this species has not been encountered by the author it is not possible to give further details. The present figures of this species are made from tracings of the drawings given by Poppe (1889).

Host. A hamster (*Cricetus cricetus cricetus* L., 1758).

Locality. Germany.

Measurements. ♂, 0·288 × 0·282 mm.; ♀, 0·4 × 0·24 mm.

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(*MS. received for publication* 28. IV. 1954.—Ed.)

THE IDENTIFICATION AND ESTIMATION OF ACETYLCHOLINE IN THREE PARASITIC NEMATODES (*ASCARIS LUMBRICOIDES, LITOMOSOIDES CARINII*, AND THE MICROFILARIAE OF *DIROFILARIA REPENS*)

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(With 6 Figures in the Text)

The experiments described in this paper were made in an attempt to show that acetylcholine is a natural tissue constituent of nematodes, this being an essential preliminary to any discussion of the possibility that acetylcholine exerts a physiological function in these animals. An approximate estimate was made of the amount of this substance present in a number of different tissues, including those rich in ganglion cells.

Baldwin (1943) and Baldwin & Moyle (1947, 1949), using *Ascaris lumbricoides*, have shown that the reactions of nematode muscle to various substances differ considerably from those of annelid tissue. Nerve-muscle preparations of *Ascaris* were found by them to be stimulated by acetylcholine in concentrations as dilute as 10^{-6} but not below. As this stimulation did not fall off with time, they concluded that their *Ascaris* preparations did not contain true cholinesterase.

Bueding (1952) detected true cholinesterase in homogenates prepared from a number of metazoan parasites including *Schistosoma mansoni*, *Litomosoides carinii*, and, in much smaller quantities, in *Ascaris lumbricoides*. The concentration of cholinesterase in schistosomes was found to be similar in amount to that of vertebrate central nervous tissue, and Bueding suggests that acetylcholine may well play a physiological role in this trematode.

MATERIALS AND METHODS

Tissue extracts were made from the following sources:

(*a*) The whole worms of *Litomosoides carinii*, a filarial nematode, the adults of which inhabit the pleural cavity of cotton rats. Collections of mixed male and female worms were used as well as batches of males and females separately.

(*b*) 'Heads' of *Ascaris lumbricoides*, a large nematode, found in the small intestine of pigs. Each 'head' consisted of the anterior 4 mm. of a worm and included the nerve ring with its associated ganglia. For preparing a tissue extract, about forty of these portions were required.

(*c*) Strips of dorsal body wall from female *A. lumbricoides*. Each strip was taken from the region immediately anterior to the genital pore, and three strips, about 5 cm. in length, were used at a time. Each piece of material consisted of cuticle,

dorsal muscle, and dorsal nerve cord, the latter being composed only of nerve fibres.

(*d*) Microfilariae of *Dirofilaria repens*, another filarial nematode, the adults of which parasitize dogs.

These extracts were assayed on preparations of leech muscle, as described by Feldberg & Krayer (1933).

All the tissues, except the microfilariae of *D. repens*, were extracted by adding N/3 hydrochloric acid, at the rate of 2 ml./g., a drop of 1:200 eserine sulphate, a small amount of universal indicator and a little sand, to a known amount of material, and then grinding for about 2 min. Where less than a gram of material was available 1 ml. of N/3 hydrochloric acid was added. After grinding, the tissue was washed into a small flask using equal amounts of distilled water and Locke's solution. It was next boiled gently for 1 min., filtered through cotton-wool, and the flask and wool were washed with a small amount of Locke's fluid. The extract was then ready for use, but it was usually kept in the cold room at 2° C. until next day. Before the extract was assayed on the leech muscle, it was neutralized with N/3 sodium hydroxide. The volume was divided into halves, one-half being made alkaline and boiled for 2 min. to destroy any acetylcholine-like substance. The latter portion was next cooled, neutralized, and used as a control. In carrying out the assay, known amounts of acetylcholine were added to this control solution, according to the method of Feldberg (1945).

The sensitivity of the leech muscle was first standardized by adding a known weight of acetylcholine in 5 ml. of Locke's solution to the leech bath and observing, on the rotating smoked drum, the contraction produced. The usual amount tried was 0·004 μg. (see Fig. 1.) Five ml. of a suitable dilution of the neutralized extract was next used, and the size of the contraction noted. This was followed by 5 ml. of the same dilution of the control. Then, known amounts of acetylcholine were added to 5 ml. of the control extract to be tested until two contractions of the control plus acetylcholine were obtained, one of which was just greater and one slightly less than that made by 5 ml. of the extract. The mean weight of acetylcholine added to the control to produce these contractions was taken to be the amount present in the wet weight of tissue dissolved in 5 ml. of the test extract, from which the weight of acetylcholine per gram of tissue was calculated.

Before an extract could be made from the microfilariae of *D. repens*, these larvae had to be collected and concentrated from a known volume of infected dog's blood. The method used was that described for trypanosomes, by Bulbring, Lourie & Pardoe (1949). For this purpose 40 ml. of venous blood were taken and added to 120 ml. of sodium citrate-saline solution (1 g. $Na_3C_6H_5O_7$, $2H_2O$/100 ml. + 0·85 g. NaCl/100 ml.), to which sufficient eserine sulphate had been added to give a concentration of 1:10,000. This was centrifuged at 3500 r.p.m. for 5 min., after which the supernatant fluid was pipetted off and replaced with 0·85 % eserinized saline. It was then centrifuged again, and the excess saline removed. The deposit consisted of densely packed blood corpuscles and microfilariae. To these, an equal amount of saline plus 8 ml. of N/3 hydrochloric acid were added. The extract was then made in the usual way except that the neutrality of the fluid was determined electrically.

The dilution of the final volume with Locke's fluid was arranged in such a way that 5 ml. contained the extract from the number of microfilariae which had been present in 1 ml. of the original blood. At the same time as the dog was bled, a count of microfilariae in 20 mm.^{3} of blood was carried out.

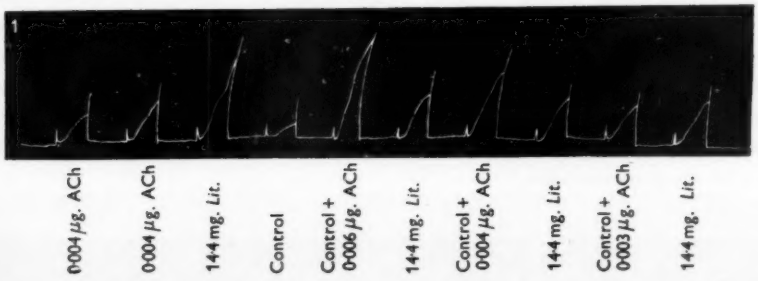
RESULTS

A. *Identification and determination of the acetylcholine-like substance in extracts of nematode tissues*

Preliminary experiments with extracts of *Litomosoides* whole worms and *Ascaris* 'heads' and body wall, showed the presence in these tissues of small amounts of an acetylcholine-like substance. Because the tracings made from *Litomosoides* extracts gave more consistent results than those made from *Ascaris* material, it was decided, when attempts were being made to identify this substance, to use these worms only.

The following results were obtained:

(1) An extract of *Litomosoides* worms caused the leech muscle preparation, previously sensitized with eserinized Locke's solution for 1 hr., to contract in a manner similar to that produced by a standard solution of acetylcholine (Fig. 1).



Figs. 1–4. Assays of tissue extracts from *Litomosoides carinii* (*Lit.*) (Nematoda Filarioidea) on the longitudinal dorsal muscle of a leech *Hirudo medicinalis*. ACh, acetylcholine; Ch.Est., cholinesterase.

Fig. 1. The record obtained when the leech muscle was sensitized with eserine for 1 hr.

(2) This contraction was very much reduced and altered in outline when the control extract (boiled in alkali) replaced the tissue extract (Fig. 1). Acetylcholine is destroyed by boiling in alkaline solution.

(3) When the tissue extract was used on a non-sensitized leech, the contraction was smaller than that which ensued after 1 hr. sensitization of the same leech preparation (Fig. 2). Eserine therefore potentiated the contraction caused by the extract. This would be the result expected if the extract contained acetylcholine itself.

(4) When the drop of eserine was omitted in preparing the extract of the worms, and this non-eserinized extract was used on a non-eserinized leech muscle, only a small contraction, similar in shape to that caused by the control extract, was

obtained. When the leech muscle was now sensitized for 1 hr. with eserinized Locke, a larger acetylcholine-like contraction was produced.

(5) When small quantities of material rich in cholinesterase* were added to 5 ml. of the extract, in an attempt to destroy the acetylcholine-like substance, it

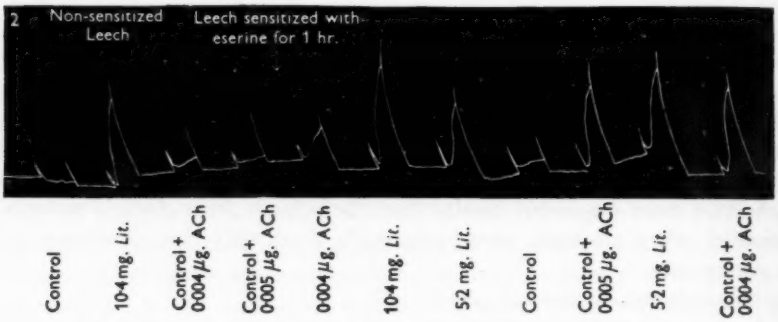


Fig. 2. The record on a non-sensitized muscle as compared with that on the same muscle after sensitization.

was found that 20 μg. acting at room temperature for 3 min. reduced the contraction. One hundred μg. acting for 5 min. resulted in a further reduction, while 200 μg. acting for 10 min. reduced the contraction and altered its shape to make it similar to that given by the control extract (Fig. 3). This showed that the acetylcholine-like substance in the *Litomosoides* extract was completely destroyed by cholinesterase, which provided additional evidence that it was acetylcholine in the extract of these nematodes.

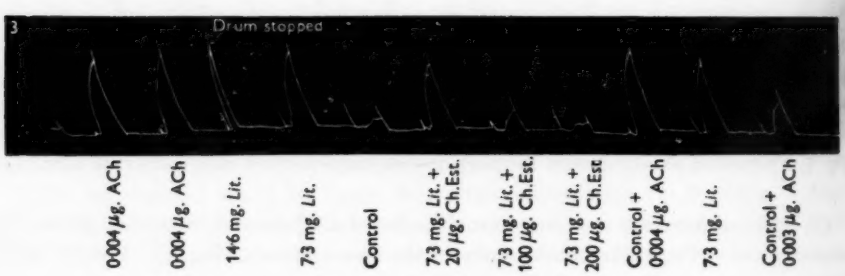


Fig. 3. A record showing the effect of different amounts of cholinesterase on the activity of the tissue extract.

The average amount of this substance (which resembled acetylcholine) in extracts made from a mixed collection of male and female *Litomosoides* worms was found to be 0·51 μg./g. wet weight (see Table 1).

* The cholinesterase material was very kindly provided by Dr Feldberg.

Table 1. *The acetylcholine content of some nematode tissues*

Material extracted	No. of assays on leech muscle	Average weight of material extracted (g.)	Average acetylcholine content in μg./g. wet weight
Litomosoides carinii, whole ♂ and ♀ worms from the pleural cavity of cotton rats	6	0·423	0·51
L. carinii, whole ♂ worms	4	0·028	0·92
L. carinii, whole ♀ worms	4	0·299	0·63
Ascaris lumbricoides, 'heads'.* Worms taken from the small intestine of pigs	6	0·113	0·39
A. lumbricoides, strips of dorsal body wall consisting of cuticle, muscle and dorsal nerve cord	6	1·145	0·025
Microfilariae of *Dirofilaria repens* taken from dog's blood	2	*c.* 0·0009	2·4

* 'Heads' of *Ascaris lumbricoides* comprised the anterior 4 mm. of the worms which included the nerve ring and its associated ganglia.

B. *A comparison of the amount of acetylcholine in male as opposed to female worms of* Litomosoides carinii

An attempt was made to gather from the pleural cavity of cotton rats, a sufficient weight of male worms from which to make an extract. But each male worm weighed only about 0·25 mg. and any one rat might not yield more than three or four male specimens, so that it was not easy to obtain a reasonable quantity before

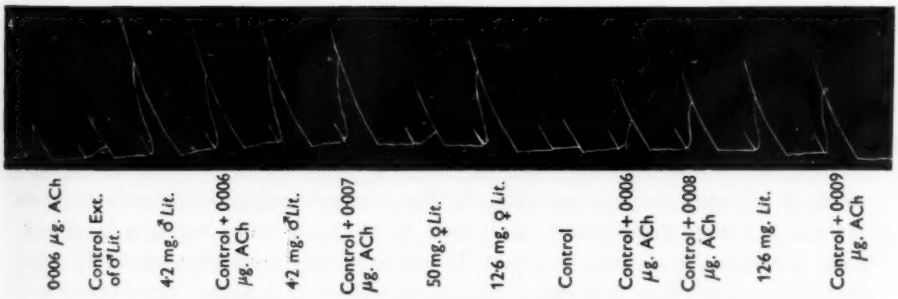

0·006 μg. ACh
Control Ext. of ♂ Lit.
4·2 mg. ♂ Lit.
Control + 0·006 μg. ACh
4·2 mg. ♂ Lit.
Control + 0·007 μg. ACh
50 mg. ♀ Lit.
12·6 mg. ♀ Lit.
Control
Control + 0·006 μg. ACh
Control + 0·008 μg. ACh
12·6 mg. Lit.
Control + 0·009 μg. ACh

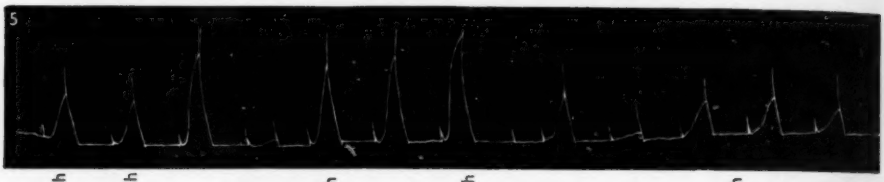
Fig. 4. A tracing showing a comparison between the acetylcholine content of male and female nematodes (*Litomosoides carinii*).

the first part of the material deteriorated. Because the average weight of a female worm was 3·6 mg., and because she is much more conspicuous, it was possible to obtain much larger weights of female than of male worms from a batch of infected rats.

The average acetylcholine content of males was found to be 0·93 μg./g., as compared with 0·63 μg./g. for the heavier female worms (Fig. 4).

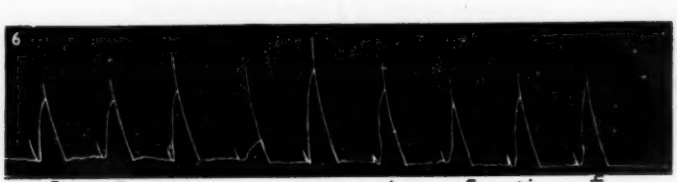
C. *Determination of the amount of acetylcholine in* Ascaris *'heads' and strips of dorsal body wall*

Whenever it was possible, the extracts made from the 'heads' and from the body wall strips were assayed, for each batch of *Ascaris* worms, on the same leech. To reduce to a minimum any possible loss of acetylcholine during dissection three of the extracts were prepared in a cold room at 2° C., after the worms had been immobilized at this temperature for 1 hr. The results did not differ from those



0·003 μg. ACh | 0·003 μg. ACh | 7·0 mg. Asc. 'heads' | Control | Control + 0·004 μg. ACh | 7·0 mg. Asc. 'heads' | Control + 0·005 μg. ACh | 6·5 mg. Asc., b.w. | Control | Control + 0·003 μg. ACh

Fig. 5. A tracing showing the difference between the acetylcholine content of nematode 'heads' (including the nerve ring), and that of the body wall (*b.w.*) in *Ascaris lumbricoides* (*Asc.*).



0·004 μg. ACh | 0·005 μg. ACh | 0·0009 mg. mf. | Control | Control + 0·003 μg. ACh | 0·0009 mg. mf. | Control + 0·002 μg. ACh | 0·0009 mg. mf. | Control + 0·0025 μg. ACh

Fig. 6. A tracing showing the approximate amount of acetylcholine in tissue extract made from the embryos—microfilariae—(*mf.*) of *Dirofilaria repens* (Nematoda Filarioidea).

obtained at room temperature (Fig. 5). The average acetylcholine content for six batches of worms was found to be 0·39 μg./g. of 'heads', and 0·025 μg./g. of body wall. Because the nervous tissue of *Ascaris* is largely concentrated in the nerve ring in the head, this provides evidence that there is a higher concentration of acetylcholine in nematode nervous tissue than in nematode body wall tissue.

D. *An estimation of the acetylcholine content of microfilariae*

For this estimation the microfilariae of the filariid nematode of the dog, *Dirofilaria repens*, were used. It was calculated from the microfilarial count that 1 ml. of the dog's blood contained 75,000 microfilariae. This was also the number of microfilariae represented by each 5 ml. of diluted tissue extract when it was placed in the leech bath. The contraction caused by the test solution was equalled by adding 0·0022 μg. acetylcholine to 5 ml. of the control extract (Fig. 6). The

75,000 microfilariae therefore contained 0·0022 μg. of acetylcholine. The weight of this number of microfilariae was found to be approximately 0·9 mg., which gives an estimated value of 2·4 μg. of acetylcholine per gram of microfilariae.

DISCUSSION

In the experiments described in this paper, it has been shown that parasitic nematodes contain small quantities of an acetylcholine-like substance. Further investigation has pointed to the fact that this is, in all probability, acetylcholine itself.

The strips from the body wall of *Ascaris lumbricoides* contained the least amount of this substance. The *Ascaris* 'heads', each of which included the nerve ring and its associated ganglia, in addition to the surrounding body wall, contained about 15 times the quantity found in the body wall alone. In the extracts made from whole *Litomosoides* worms, the amount of acetylcholine was somewhat larger than that which was found in the *Ascaris* 'heads'. At first it was thought that the *Ascaris* preparations might possibly have suffered loss of acetylcholine during the few minutes which were taken to dissect out and weigh the tissue. In the case of the *Litomosoides* worms this possibility did not arise, because they could be kept whole and alive for 2–3 hr. before they were placed in dilute hydrochloric acid. When, however, the preparations of *Ascaris* were made at 2° C., no further gain in the acetylcholine content was detected. Another possible reason why the whole *Litomosoides* worms contained more acetylcholine per gram than the *Ascaris* 'heads', was that the mature female *Litomosoides* contained a large number of embryos (microfilariae) which would, a short time later, find their way into the bloodstream of the host. They are actively motile, and by analogy with trypanosomes (Bulbring *et al.* 1949) and schistosomes (Bueding, 1952) might be expected to contain appreciable amounts of acetylcholine. When extracts were made from microfilariae of another species of nematode, the acetylcholine content was found to be higher than that of the other tissues assayed. This finding supported the view that much of the acetylcholine present in the *Litomosoides* extract might have originated from the microfilariae in the females.

However, when separate extracts were made of male and female *Litomosoides* worms, the males were found to contain, if anything, slightly more acetylcholine than the females, weight for weight, in spite of the microfilariae in the latter. This is probably due to the fact that the males have proportionately more nervous tissue than the females, because they are much smaller and they have some additional nerve ganglia, which the females do not possess at all.

Acetylcholine is found very commonly in living tissues, small amounts occurring in plants as well as in the muscles and alimentary canals of many different classes of animals. Most vertebrate tissues contain some acetylcholine, in quantities varying from 0·05 to 30 μg./g. of material (Chang & Gaddum, 1933). The larger amounts are found in the nervous tissues. Among invertebrates, the cerebral ganglia of the octopus (Bacq, 1947) and various insect ganglia have been shown to be the richest sources. The muscles of molluscs and annelid worms contain small amounts (Prosser, 1946; Katz, 1949).

The mere presence of acetylcholine in an animal means very little by itself. Bueding (1952), however, has recently shown the existence of specific acetylcholine-esterases in another group of parasitic metazoa, the schistosomes, as well as in *Litomosoides carinii*, and to a much smaller extent, in *Ascaris lumbricoides*. This increases the likelihood that acetylcholine does, in fact, play a part in nematode physiology.

SUMMARY

1. Extracts made from whole nematode worms, *Litomosoides carinii*, and from *Ascaris* 'heads' and body wall tissue, contain a substance similar to acetylcholine.

2. In the case of extracts from *Litomosoides* whole worms, this substance was shown to be probably acetylcholine itself.

3. Tissue from the anterior end of *Ascaris* (including the nerve ring), contains about 15 times as much acetylcholine as the body wall preparations; i.e. 0·39 μg./g. as compared with 0·025 μg./g. of wet weight.

4. There appeared to be rather more acetylcholine present in the *Litomosoides* males than in the females; 0·92 μg./g. as compared with 0·63 μg./g. of wet weight.

5. The microfilariae of the filariid nematode of the dog, *Dirofilaria repens*, contain as much as 2·4 μg./g. of acetylcholine. In this respect it resembles some other motile parasites of the blood, such as trypanosomes.

In conclusion I should like to thank Dr F. Hawking, in whose department this work was done, for suggesting the problem and for providing a supply of nematode material from experimental infections. I am also much indebted to Dr W. Feldberg for his valuable help and advice. Miss J. Selby and Mr R. Blakemore gave technical assistance.

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THE ADULT AND DIPLOSTOMULUM STAGE (*DIPLOSTOMULUM PHOXINI* (FAUST)) OF *DIPLOSTOMUM PELMATOIDES* DUBOIS AND AN EXPERIMENTAL DEMONSTRATION OF PART OF THE LIFE CYCLE

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(With Plate V and 10 Figures in the Text)

Dr E. G. Healey of this department, while working on colour change in the minnow (*Phoxinus phoxinus* (L.)), drew the attention of the writer to the presence in the brain of large numbers of trematode larvae. The fishes were obtained from Fron Goch Pool a few miles inland from Aberystwyth, and of several hundred examined subsequently every one was found to be infested. The parasite was identified as *Diplostomulum phoxini* (Faust, 1919). Mataré (1909) first described but did not name the worm from the brain, spinal cord, cranial cavity and eyes of the minnow in Switzerland and Munich. It was referred to the genus *Tylodelphis* by André (1918) and named *Tetracotyle phoxini* by Faust (1919). It was mentioned by Mathias (1925), and Ashworth & Bannerman (1928) first reported it from the minnow in Britain having found it in fish in a stream near Edinburgh, in Loch Lubnaig, at Reading in Berkshire, and at Radlett in Hertfordshire. It has not been recorded from any other fish host and up to the present nothing of its life history has been known.

Larvae of a similar type have been reported from many other species of fresh water fishes. The oldest known is *Diplostomulum spathaceum* (Rud.) described by von Nordmann (1832) as *Diplostomum volvens*, from the eyes of five species of fishes in Europe. Zandt (1924) listed this species from twenty-two host fishes including *Phoxinus laevis* (= *P. phoxinus*) and Hughes (1929*a*) from the eyes of a large number of fishes including *Leuciscus phoxinus* (= *Phoxinus phoxinus*) in North America. It has not been recorded from the minnow in this country, but was found by Rushton (1937, 1938) and later by Baylis (1939) in the eyes of the Rainbow trout in Britain. As pointed out by Hughes (1929*a*) *Diplostomum*, being a generic name, cannot be used for a larval group. He accepted the suggestion made by Brandes (1888, 1890) that the name *Diplostomulum* should be used as a group name for larvae resembling *Diplostomum volvens* and which occur in the eyes, brain and spinal cord of cyclostomes, fishes and Amphibia. The name Tetracotyle has been given to somewhat similar larvae which encyst in the bodies of molluscs, leeches and all classes of vertebrates. The specific name given to a larva of the *Diplostomulum* group will disappear when its adult stage is known; thus *Diplostomum volvens* v. Nordmann has become *Diplostomulum spathaceum* (Rud.) Hughes 1929*a*, the larval stage of *Diplostomum spathaceum* (Rud.), and *Diplostomulum phoxini* (Faust) becomes, as will be shown later in this paper, *D. pelmatoides* Dubois, the larval stage of *Diplostomum pelmatoides* Dubois.

Two other species of these larvae described by von Nordmann are *Diplostomulum clavatum* and *D. brevicaudatum*, but both these may be synonymous with *D. spathaceum*. *D. lenticola* (v. Linstow, 1878) referred to by Braun (1894), Mataré (1909) and Faust (1919) from *Abramis vimba* (L.) is another species which may be synonymous with *Diplostomulum spathaceum*.

Another well-known form is *D. petromyzi-fluviatilis* (Diesing) first found by Müller in 1839 in the fourth ventricle of *Lampetra fluviatilis* (L.). It has been recorded by several writers, namely Diesing (1850), Cobbold (1860), Gulliver (1872) and Brown (1899) and occurs in Britain and other European countries.

The *Diplostomulum* type of larva belongs to the Strigeida. In very few cases has the life history of a member of this group been worked out. In those which have the first intermediate host is a gastropod mollusc from which fork-tailed cercariae emerge. These penetrate into the body of the second intermediate host, a fish or amphibian in which they develop into a *Diplostomulum*, the metacercaria stage. The definitive hosts are mainly fish-eating birds, the *Diplostomulum* developing into the adult worm in the intestine of the bird.

The descriptions of *D. phoxini* (= *D. pelmatoides*) given by Mataré (1909), and Ashworth & Bannerman (1928) are adequate in all respects except for the flame-cell system which has not been described by either. It was decided, therefore, to examine the parasite more closely in an attempt to work out the arrangement of the flame cells and also, by means of feeding experiments with a suitable bird host, to obtain experimentally the adult stage of the parasite. To obtain live specimens of the worm the fish were anaesthetized by scattering crystals of eurethane into the water in a small vessel containing the fish. Immediately after death the fish were washed and decapitated, the head then being examined in normal saline. The brain was removed by dissection under a binocular and living specimens of the worm removed from this by opening it along its length. Living worms were mounted in saline, varying degrees of pressure were exerted on the cover-glass and they were examined with a $\frac{1}{6}$ in. and a $\frac{1}{12}$ in. oil-immersion objective. By this means and with repeated examination of a very large number of specimens most of the anatomical details could be seen. Sections of the head and brain of infested minnows were cut so that the exact distribution of the parasite could be ascertained. For this purpose fish, after being killed with eurethane, were fixed whole in Bouin's fixative in which they were left for about 8 weeks. Transverse and sagittal sections of the head were cut at 10μ in thickness and stained with Ehrlich's haematoxylin and Orange G. Normal heads obtained from uninfested fishes from a different locality were treated in the same way and sectioned for comparison. In some cases the brain was removed from infested fishes, fixed in Carnoy's and sectioned separately, but there was no special advantage in this over sectioning the entire head. The latter was really more satisfactory, as it showed the presence of the larvae in the cranial cavity and eyes in addition to the brain and spinal cord.

By far the majority of larvae occur in the brain itself. An infested brain compared with a normal one (Pl. V, figs. 1–3) is swollen in the region of the mid-brain, cerebellum and medulla and the lobi inferiores are more prominent. When the brain is sectioned most of the worms appear to be in the cavities of the brain,

being particularly abundant in the 4th ventricle (Pl. V, figs. 2, 3). In an entire brain, immediately it is exposed, a mass of them can be seen crowded in the 4th ventricle under the pia mater where they undergo slow rhythmic movements of contraction and expansion. Brown (1899) stated that the brain of ammocoetes is 'bulged out' by similar larvae which may be 'squirted 18 in. into the air when the roof of the brain is divided suddenly with a sharp knife'. Other parts of the brain in which they occur are the aqueductus sylvii, the optic lobes and 3rd ventricle, the lobi inferiores, occasionally the corpora striata and underneath the epithelium covering the surface of the brain. None has been found in the olfactory lobes. The larvae occur in varying numbers in the spinal cord especially in the dorsal part of the region following the medulla oblongata. Fig. 2 (Pl. V) is a sagittal section through the brain of a fish which was showing no symptoms of infestation. About a hundred larvae are apparent in one section and they occur mainly in the 4th ventricle, aqueductus sylvii, optic lobes, 3rd ventricle and a small number in the spinal cord. There was none in the lobi inferiores or corpora striata. Figs. 4–6 (Pl. V) show a brain in which the infestation was slightly heavier but still the fish showed no signs of distress. The parasites can be seen in the aqueductus sylvii (Pl. V, fig. 4), in the early part of the spinal cord around the root of the first spinal nerve (Pl. V, fig. 5) and further back in the spinal cord (Pl. V, fig. 6). In spite of infestations of this degree the fishes live quite comfortably for long periods in captivity and appear to suffer no inconvenience. It is not until they are killed and the brain examined that there is any indication of infestation. Fig. 3 (Pl. V) is a sagittal section through the head of a fish which was dying, presumably as a result of infestation. It exhibited intermittent swimming movements between which it floated up to the surface of the water turning on to its side. It was killed and dissected, and as no other cause for these symptoms could be found it was reasonable to suppose that they were due to damage to the brain and spinal cord caused by the contained parasites. A larger number of worms was present than in any other specimen sectioned, 143 could be counted in a single section. They are more extensively distributed than in the previous sections extending into the lobi inferiores and the corpora striata; they are also more abundant in the spinal cord. A small number of fish showing similar symptoms was killed and examined, the brain being removed, teased apart and in each case the contained worms counted by removing them singly with a pipette. The largest number found in this way in a single brain was 1296, and probably this does not include all the individuals present, some at least being unavoidably lost during the process of dissection. Fish from the same locality showing no symptoms were examined in the same way and were found to contain up to 600 worms in the brain. Mataré (1909) suggested a maximum of 500, and Ashworth & Bannerman (1928) quoted several dozen in a brain and a maximum of 247, none occurring in the spinal cord.

From an examination of fish showing various degrees of infestation the first part of the brain to be invaded appears to be the 4th ventricle from where with increase in numbers the worms extend forwards through the aqueductus sylvii into the 3rd ventricle and optic lobes and backwards into the spinal cord. Only in very heavily infested specimens do they enter the lobi inferiores and the corpora striata.

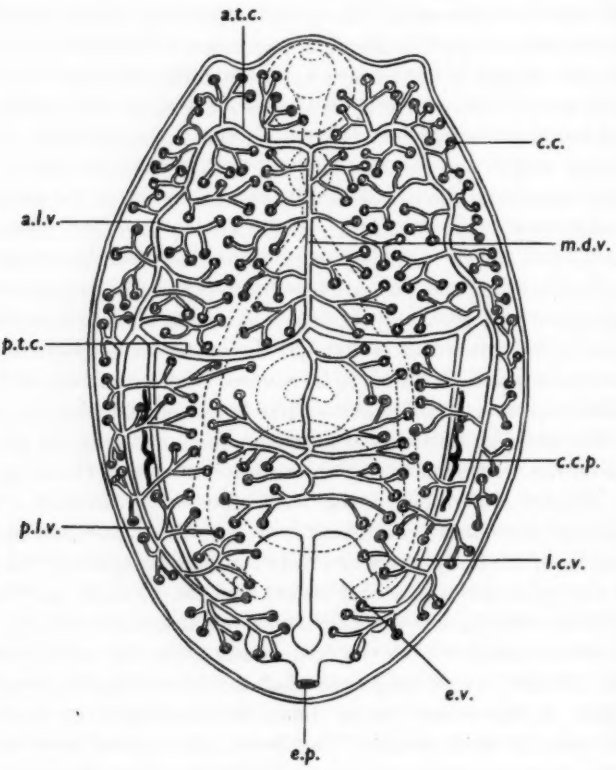
Mataré (1909) never found them in these two latter locations. André (1918) stated that they are almost always found in the 4th ventricle and up to the aqueductus sylvii. His was not a heavy infestation as he found a total of only thirty worms in an incomplete series of sections. The worms also occur in the cranial cavity and in the fissures between the various lobes of the brain. On closer examination the worms do not appear to be free in the cavities of the brain but seem originally to be located under the epithelium lining the cavities. This they raise up, there being an apparent proliferation of vacuolated tissue underneath in which the worms lie each in its own little compartment, so that they do not touch one another (Pl. V, figs. 2, 3 and 5). Fig. 5 (Pl. V) shows this clearly where the posterior end of the 4th ventricle is almost obliterated by this tissue. Similarly, when the worms occur under the meninges this layer is raised up but the vacuolated tissue is not as abundant as in the ventricles (Pl. V, figs. 2, 3). Mataré (1909) stated that there was no injury to the brain or meninges close under which the worms are situated. Sometimes the worms are more deeply embedded in the tissues of the brain or spinal cord (Pl. V, figs. 3, 6). There is, however, no evidence of movement of the worms through the tissues. Presumably they reach the brain via the blood stream. There are fairly large blood vessels entering the brain but no worms have been found in them. Blochman (1910) reported the death of aquarium fishes as a result of the entry of *Cercaria flexicauda* La Val. into *Gobio fluviatilis* and *Rhodeus amarus* which died 3 hr. after exposure to the parasites. A post-mortem examination showed worms in the pericardial cavity, arteries, eyes, lens and brain. Baylis (1939) reported the death of Rainbow trout due to cerebral haemorrhages caused by large numbers of migrating cercariae of *Diplostomum spathaceum*. If, however, the infestation is gradual over a period of time there are probably insufficient larvae entering together to cause fatal haemorrhages. Davis (1936) suggested that injury to the central nervous system of *Cotastomus commersonii* by *Diplostomulum flexicaudum* may cause paralysis and promote a fatal haemorrhage.

D. phoxini (*D. pelmatoides*) has also, in the present instance, been found in the eyes of the host. They occur, though not in very large numbers, in the vitreous body and lens. Those in the vitreous body exhibit slight activity while those in the lens are motionless, usually elongated and lie between the concentric lamellae of lens fibres in the outer cortical region of the lens. They can, with care, be dissected out from the lens when they move sluggishly as do those from the brain. Mataré (1909) recorded them from the eyes, but André (1918) found them in the brain only, as also did Ashworth & Bannerman (1928). The majority of *Diplostomulum* larvae have been recorded more frequently from the eyes of the host than from the brain. von Nordmann (1832) recorded up to 400 specimens of *Diplostomum volvens* (= *Diplostomulum spathaceum*) in the eyes of *Cyprinus erythrophthalmus*. He found them in the aqueous body, between the cornea and iris and in the lens and stated that they might produce cataract. Butler (1919) reported the same species in the anterior and posterior chambers, on the iris and in the pigmented tissue of the choroid, but mostly in the vitreous body of seven species of fishes from Douglas Lake, Michigan. Baylis (1939) recorded *Diplostomulum spathaceum* from the eyes of the Rainbow trout, some fish being blind in both eyes as a result. This blindness,

he suggests, probably favours the completion of the life cycle of the worm by making the fish easier prey to birds. Hughes (1929*b*) found from one to fifty-five specimens of *D. scheuringi* in the vitreous body but not in the lens of various freshwater fishes in North America.

On removal from the brain the worms do not display any marked activity but creep sluggishly or remain stationary for some time. They will live for several days in saline at room temperature. They are opaque and whitish in colour, the two regions of the body being distinguishable, the anterior spoon-shaped concave ventrally and trilobate anteriorly, the posterior bluntly conical or rounded. The shape varies somewhat with the degree of expansion and contraction and the distinction into two regions is not always apparent under pressure of a cover-glass. The average measurements of a number of specimens agree very closely with those given by Ashworth & Bannerman (1928). In the expanded state they measure 0·344 × 0·13 mm. and in the contracted 0·247 × 0·215 mm. Mataré's (1909) specimens were only about half the size of those of the writer and of Ashworth & Bannerman, measuring only 0·20 × 0·15 mm. André (1918) gave the measurements as 0·23 × 0·15 mm., but stated that they were taken from contracted specimens and must be less than in living specimens. These discrepancies in size, therefore, are not regarded as being of very great importance. The cuticle is 0·004 mm. thick. Mataré (1909), Ashworth & Bannerman (1928) and the writer have observed the peculiar appearance of the surface. It seems to be covered with tiny facets and in profile to contain vertical striations looking almost like little spines which do not penetrate the surface. The same condition is apparent in the adult stage of the worm. The oral sucker is on the ventral surface at the anterior extremity (Text-fig. 1, 2), and is rounded or slightly elongated in the antero-posterior direction. It measures 0·046 × 0·041 mm.; the mouth is directed forwards. The ventral sucker, situated at about the middle of the body, is transversely oval and measures 0·043 × 0·050 mm. It is almost the same size as the oral sucker and can be sunk into the body. On either side of the oral sucker are the lateral organs, each situated on a slight prominence which together with the oral sucker gives the trilobed shape to the anterior end. Mataré (1909) suggested that the lateral organs may function as organs of taste, as the cuticle lining them, though similar in structure to that covering the body, is much thinner. They have, also, a good nerve supply. There appear to be no adhesive glands associated with them, but they may be suctorial as powerful groups of muscles are inserted into the base of each and pass backwards spreading out fanwise to their points of origin in the longitudinal muscles of the body wall dorsally and ventrally. These muscles are even more powerfully developed in the adult. The hold-fast organ is close behind the ventral sucker. It is transversely oval, measuring 0·066 × 0·083 mm., and the deep lumen opens on the surface by an antero-posterior slit, which may have from three to seven radii (Text-fig. 2). Mataré stated that there were papillae around the aperture but it is possible that these are really the projections between the radii. The lumen is lined with cuticle and there is a muscular wall surrounded by gland cells. Muscle fibres arising from the body wall are inserted into the adhesive organ and control the size of the lumen. The whole structure may at times be everted. There is a small prepharynx,

short oesophagus and the intestinal limbs terminate dorsally to the excretory vesicle at about half-way along its length. The amount of inward curvature at the posterior extremity of the intestinal limbs depends on whether the excretory vesicle is full or empty, the curvature being usually more marked in the latter case. Mataré described the contents of the gut as forming a dark granular mass which is probably derived from blood. Ashworth & Bannerman also suggested that the food is blood and cerebral fluid.

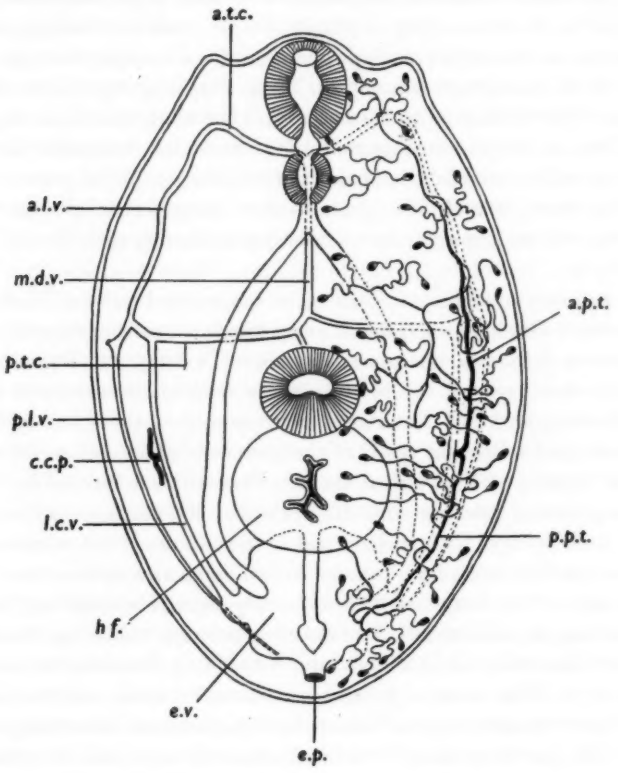


Text-fig. 1. *Diplostomulum pelmatoides* Dubois (*D. phoxini* (Faust)). Reserve bladder, dorsal view.

The musculature and nervous system have been described by Mataré and Ashworth & Bannerman. There is no disagreement in the case of specimens observed by the writer, and so no account of them will be given here.

The excretory vesicle is situated in the posterior part of the body, occupying the region between the hold-fast organ and the posterior extremity, its anterior end overlapping dorsally the posterior margin, of the hold-fast organ (Text-figs. 1, 2). It is bilobed and communicates posteriorly by a short canal with the excretory

pore on the dorsal side just in front of the posterior extremity. The excretory pore is surrounded by circular muscles forming a sphincter. There are two sets of excretory tubules, namely, the primary excretory apparatus or flame cell system (Text-fig. 2) and the secondary excretory apparatus, the so-called reserve bladder (Text-fig. 1). The reserve bladder is in all essential points similar to that in *Diplostomulum spathaceum* described by Claparède (1858) and Fraipont (1880), *D. browni* described by Hughes (1929*a*) and *D. ambystomae* described by Rankin & Hughes (1937). It consists on either side of a lateral collecting vessel which extends from



Text-fig. 2. *Diplostomulum pelmatoides* Dubois (*D. phoxini* (Faust)). Flame cell system, ventral view.

the point where it opens into the excretory vesicle forwards to the level of the anterior margin of the ventral sucker (Text-figs. 1, 2). Here it bifurcates, giving rise to an antero- and postero-lateral vessel. The postero-lateral vessel runs backwards near the margin of the body to terminate blindly in the region of the excretory vesicle. In this respect it resembles the description given by Ashworth & Bannerman (1928). Mataré (1909) described this vessel as opening into the excretory vesicle, but it has not been observed to do so here or in any other described

Diplostomula. The antero-lateral vessel continues forwards, laterally, to the level of the oral sucker and then turns towards the centre of the body, forming the anterior transverse commissural vessel. Where the vessels from the right and left sides meet in the middle line there is formed the median dorsal vessel which runs posteriorly and terminates blindly dorsally to the hold-fast organ (Text-fig. 1). This vessel is also joined by a pair of posterior transverse commissural vessels situated in front of the ventral sucker and arising from the lateral collecting vessels shortly before they bifurcate. From all these vessels except the lateral collecting vessels and posterior transverse commissural vessels arise numerous short ducts which may divide dichotomously or remain simple, each terminating in a blind sac which is more or less spherical in shape and contains a conspicuous highly refractile spherical or ovoid calcareous concretion. These vary slightly in size, the spherical ones measure 0·006–0·01 mm. in diameter, and the oval, which are larger, 0·012 × 0·008 mm. The number and arrangement of the secondary branches varies from one individual to another, the variation being most noticeable at the posterior extremity of the median dorsal vessel. The general effect, however, in all cases is that the calcareous concretions appear to be more or less uniformly distributed in the dorsal half of the body.

The only primary excretory apparatus so far worked out for *Diplostomulum* is that by Rankin & Hughes (1937) for *D. ambystomae*; in certain respects it resembles the arrangement found in the present species (Text-fig. 2). There are fifty-two flame cells on either side of the body together with their system of ducts. Each common collecting trunk of the primary system is very short and opens on either side into the lateral collecting vessel of the reserve bladder a short distance behind the posterior margin of the ventral sucker. This trunk is formed by the union of anterior and posterior primary collecting tubules. The posterior primary collecting tubule runs back parallel with and ventral to the tubules of the reserve bladder. It receives in series five secondary tubules from five groups each of four flame cells. Each secondary tubule branches dichotomously twice, the resulting four branches each terminating in a flame cell. The anterior primary collecting tubule runs forwards and divides into two at a level about half way between the ventral sucker and the pharynx. The anterior branch continues forwards and receives in series three secondary tubules, each of which by dichotomous branching ends in four flame cells. The posterior branch turns backwards and runs between the other lateral vessels and the ventral sucker. It receives in series five secondary tubules each again by dichotomous branching being connected with a group of four flame cells.

The flame cell formula, therefore, can be expressed as follows:

$$2[\{(4+4+4)+(4+4+4+4+4)\}+(4+4+4+4+4)]=104,$$

or in a simplified form as

$$2[\{(12)+(20)\}+(20)]=104.$$

The number of flame cells, fifty-two pairs, is greater than in *D. ambystomae* in which there are forty-four pairs. The flame cells in this type of larva are extremely difficult to find owing to the calcareous concretions which are so liberally distributed

throughout the body. Fraipont (1880) found five groups of flame cells in *Diplostomum volvens*, the distribution of which coincides with some of those seen by Rankin & Hughes (1937) and the writer in the species examined by them. Fraipont stated that the ducts from the primary system open into the larger vessels at two points, namely, near the middle of the length of the antero-lateral vessels and near the opening of the lateral collecting vessels into the excretory vesicle. He suggested the presence of a third opening in the region of the bifurcation of the lateral collecting vessel. This differs from the arrangement found by the writer for *Diplostomulum phoxini* and by Rankin & Hughes for *D. ambystomae* where only one opening exists on either side.

In many ways *D. phoxini* (=*D. pelmatoides*) resembles *D. spathaceum* (Rud.) and *D. petromyzi-fluviatilis* (Diesing). It is about the same size as the former, but the dimensions of the suckers are different. The ventral sucker in *D. spathaceum* is about twice the diameter of the oral sucker and is situated slightly anteriorly to the middle line, there being an interval between it and the adhesive organ. The excretory vesicle and excretory tubules of the reserve bladder are almost identical in arrangement. In *D. petromyzi-fluviatilis* the arrangement of the internal organs is very similar to that described here. The main differences are that in the former the intestinal limbs terminate immediately behind the adhesive organ close to the middle line. The transverse anastomoses of the reserve bladder are only feebly marked and the excretory vesicle consists of two widely separated portions opening by a short common duct to the exterior.

Neither Mataré (1909) nor Ashworth & Bannerman (1928) made any attempt to elucidate the life cycle or to find any other stages in the life history of *Diplostomulum phoxini*. Other writers who have mentioned the worm, namely, Faust (1919), André (1918), Mathias (1925) and Hughes (1929*a*) have confined their remarks to the anatomy and occurrence of the metacercaria.

As already indicated, larvae of the *Diplostomulum* group belong to the genus *Diplostomum*, the species of which are parasitic, mainly, in the intestine of fish-eating birds. Only in two species of the genus is the life cycle known, namely, *Diplostomum spathaceum* (Rud.) and *D. flexicaudum* (Cort & Brooks) van Haitsma. In the former Braun (1894) reported on feeding experiments by the Ehrhardt brothers which demonstrated that *Diplostomulum spathaceum* (Rud.) (=*Diplostomum volvens* v. Nordmann) developed into *Diplostomum spathaceum* (Rud.) in the intestine of *Larus ridibundus ridibundus* L. and *Sterno hirundo hirundo* L. It was Szidat (1924) who completed our knowledge of the life cycle when he showed that *Cercaria Diplostomuli spathacei* (Rud.) (=*Cercaria C.* Szidat (1924) and *C. helvetica* XIII Dubois (1929)) from *Lymnaea stagnalis* developed into the *Diplostomulum* stage in the lens of the eye of various freshwater fishes. It has been recorded by Dubois (1938) from forty-four different host fishes. *Diplostomum flexicaudum* (Cort & Brooks) is a parasite in the adult stage of *Larus argentatus argentatus* Pont. The cercaria of this worm, *Cercaria flexicauda*, was described by Cort & Brooks (1928) from *Lymnaea emarginata angulata* Sowerby. The metacercaria stage was described independently by Hughes & Berkhoult (1929) as *Diplostomum gigas* from the lens of the eye of *Catastomus commersonii* (Lacépède)

and the life history was eventually worked out experimentally by van Haitsma (1931).

Another life cycle, which has been worked out in part, is that of *Diplostomum huronense* (La Rue, 1927*a*) by Hughes (1929*b*) from *Larus argentatus* Pont. The metacercaria *Diplostomulum huronense*, from the lens of the eye of *Percopsis omiscomaycus* Walbaum and *Perca flavescens* Mitchill in Lakes Douglas and Oneida, develops into *Diplostomum huronense* when fed to gulls. The first larval stage of this parasite is not yet known. Butler (1919) fed young herring gulls on dead fish from Douglas Lake, Michigan, and the birds became heavily infested with trematodes. Larvae of the *Diplostomulum* type were found in large numbers in the eyes of fishes in the lake, but the relation of the larval forms in the fishes to the adults in the gulls though suspected, was never proved.

In the present instance the adult stage of *Diplostomulum phoxini*, namely *Diplostomum pelmatoides* Dubois, has been obtained by feeding infested fish to domestic ducks. The first larval stage, the cercaria, is not yet known, but attempts will be made in the near future to complete the life cycle.

It seemed almost certain that the adult would belong to the genus *Diplostomum* v. Nordmann and that the final host would be a fish-eating bird. The choice of a species of bird host for experimental purposes was influenced by the known natural hosts of species of the genus *Diplostomum*. According to Dubois (1944) these occur in eight groups of birds, being most frequently found in the Lari which serve as hosts for six species and the Anseres in which five species are known. A very small number occurs in other groups of birds, namely, Charadrii, Alcae, Colymbi, Staganopodes, Accipitres and Striges. Of the five species occurring in the Anseres, namely, *Diplostomum mergi* Dubois, *D. parviventosum* Dubois, *D. pusillum* (Dubois), *D. pelmatoides* Dubois and *D. orientale* Yamaguti, nothing is known of the life history. Of those in the Lari the complete life cycle of two is known, namely, *D. spathaceum* (Rud.) and *D. flexicaudum* (Cort & Brooks), but nothing is known of the life cycles of *D. indistinctum* (Gub.), *D. baeri* Dubois and *D. commutatum* (Dies.).

It was conceivable that *Diplostomulum phoxini* might be the metacercaria of one of the species from the Lari or Anseres in which nothing of the life cycle was known. The easiest available host for experimental purposes was the domestic duck, and so it was decided to attempt feeding experiments with this species in the first instance. Five day-old ducklings were obtained on 3 July 1953, and were housed by Mr L. E. Hughes, Veterinary Investigation Officer in the veterinary laboratory at Aberystwyth. The actual feeding of the birds was done by Mr Hughes in the presence of the writer, who is most grateful for his assistance. After their arrival the birds were kept in their cages for 3 days to allow them to feed and to become used to their surroundings. On 6 July duckling (1) was given forty eyes taken from 20 infested minnows; duckling (2) and (3) ten infested brains each, and duckling (4) the entire heads of five infested minnows. They were labelled, returned to their cages, and fed in the usual way. Duckling (5) served as control. Ducklings (1) and (2) were killed with chloroform on 10 July, 4 days after infestation. The alimentary canal was removed and opened in measured portions in saline using a binocular. Duckling (1), which had received the infested eyes, contained two

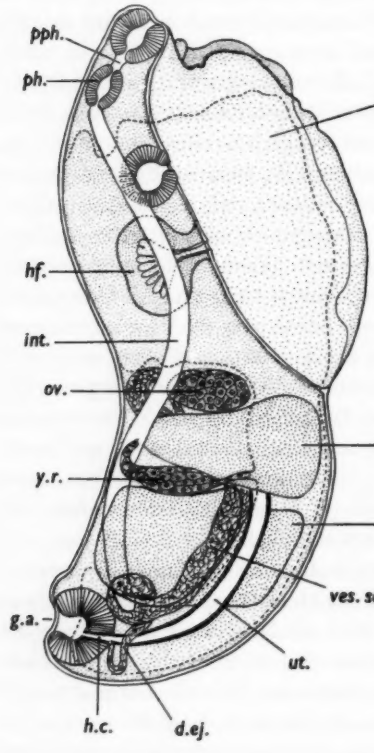
specimens of an adult *Diplostomum* in the first 8 in. of the intestine, and duckling (2), which had been given ten infested brains, thirty, four in the first 8 in. and the remainder in the second 8 in. of the intestine. Ducklings (3) and (4) were killed on 11 July, 5 days after infestation and examined in the same way. In duckling (3), fed with ten infested brains, 542 worms were recovered from the first 16 in. of the intestine and in duckling (4), which had been given entire heads, nine were found. The control duckling, killed at the same time, was free from infestation.

On removal from the host the worms were washed in saline and fixed either in Gilson's fixative or 5% formaldehyde. Some specimens were mounted whole and stained with Coelestin Blue, while others were embedded for sectioning. As the worms were extremely small it was found to be advantageous to stain them in Ehrlich's haematoxylin first so that they could be seen and orientated when making the wax blocks. Horizontal and sagittal sections were cut, some at 5μ and others at 10μ in thickness, and stained with Ehrlich's haematoxylin and Orange G.

The adult specimens were identified by means of Dubois's key (1938, 1951, 1953) as *D. pelmatoides* Dubois (1932). The metacercaria stage, therefore, should now be known as *Diplostomulum pelmatoides* Dubois and not *D. phoxini* (Faust). Dubois recorded the species as having been found on one occasion only when eleven specimens were obtained from the intestine of *Mergus merganser merganser* L. As far as the writer has been able to ascertain the adult stage has not been recorded previously in this country and its normal host here is not yet known. All four species of *Diplostomum* recorded in Europe from Anseres occur only in *Mergus merganser merganser* except for *Diplostomum mergi* which is found also in *Mergus serrator* L. It has not been possible to obtain any specimens of *M. merganser merganser* for examination as yet; the bird occurs in the Dovey Estuary during the winter months but has never been reported from the pool where the infested minnows are found. Some other bird may serve as definitive host in this country as the following have been reported from the pool in question, namely, Heron, Mallard, Cormorant, Great Crested Grebe, Little Grebe, Coot, Moorhen, Black-headed Gull, and as a winter visitor the Tufted Duck. All these birds include fish in varying quantities as part of their diet. Two specimens of the Tufted Duck (*Nyroca fuligula*(L.)) and one of the Teal (*Querquedula crecca crecca* (L.)) from a neighbouring pool have so far been examined with negative results. It is hoped that some of the above-named birds will be obtainable in the not too distant future and that the natural host of the parasite will be found. As all the minnows caught in the pool during the last 3 years have been infested there must be a constant source of infestation from some bird frequenting the pool.

Diplostomum pelmatoides was so named by Dubois (1932) because, in outline, it resembles the sole of a foot (Text-fig. 4). The total length varies from 0·614 to 0·937 mm. and the average length of ten specimens is 0·734 mm. Dubois gave the length as 0·7–1·0 mm. There is no strongly marked transverse constriction between the anterior and posterior parts of the body as is usually the case in members of this genus, though both regions are easily recognizable. The anterior part of the body, which is adapted morphologically for the fixation of the parasite, is more or less oval in outline measuring 0·322–0·437 mm. in length and 0·341–0·448 mm. in

maximum breadth. The posterior part, which contains the reproductive organs, is shorter than the anterior. It measures 0·291–0·416 mm. in length and 0·223–0·360 mm. in breadth and is more or less cylindrical in shape, tapering slightly to a broadly rounded extremity. On the dorsal side near the posterior end opens the genital atrium (Text-figs. 3, 5, 9 and 10). At the junction between the anterior and posterior parts of the body there arises on the ventral side a deep fold which continues forwards on either side along the lateral margins of the anterior half of the body (Text-figs. 3–5). This projects inwards over the ventral surface so that the body has, in lateral view, the appearance of a 'horn of plenty', as noticed by Dubois with a large oblique aperture directed antero-ventrally (Text-fig. 3). This illusion is



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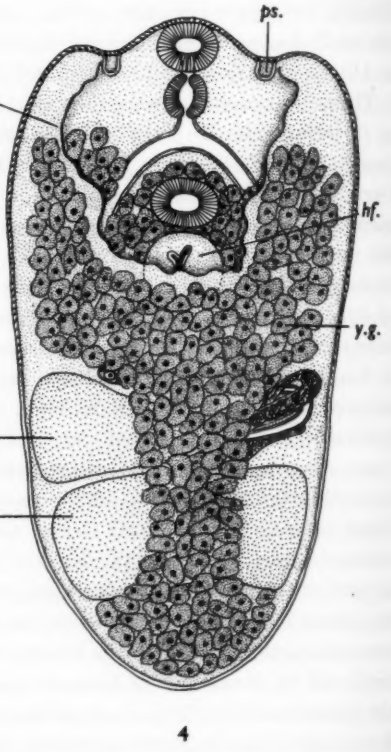

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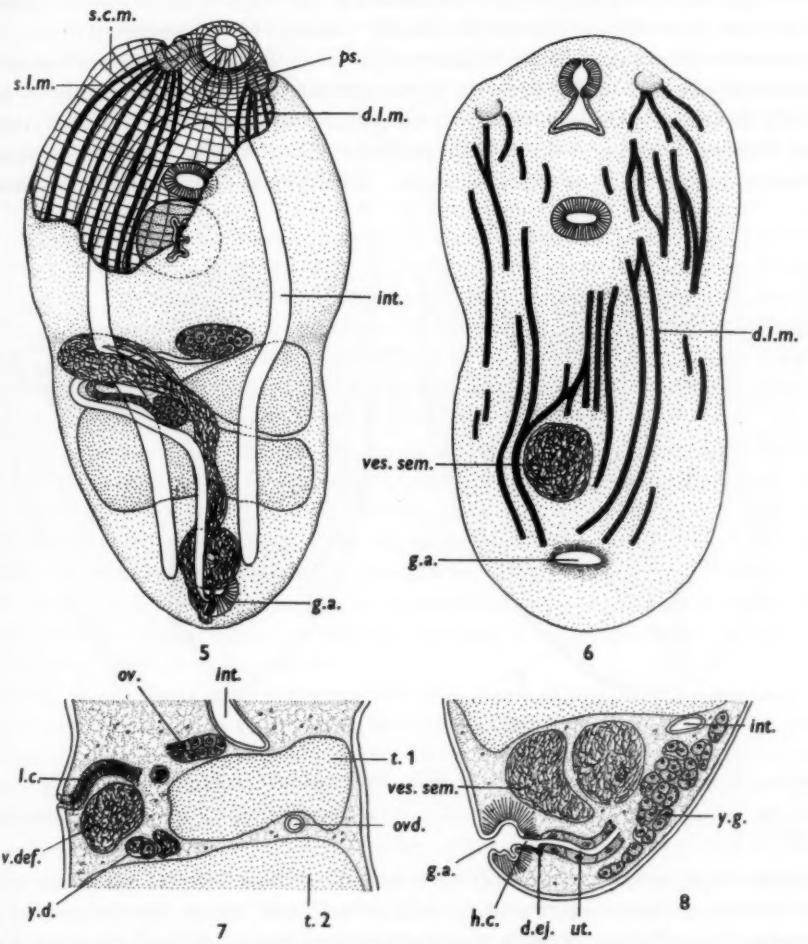
Text-fig. 3. *Diplostomum pelmatoides* Dubois from the right side. Area occupied by yolk glands indicated by dotted line.

Text-fig. 4. *D. pelmatoides*, ventral view showing ventral fold and distribution of yolk glands.

emphasized by the fact that the posterior part of the body, when seen in lateral view, often curves dorsally. The anterior extremity is trilobed (Text-fig. 4), the median rounded lobe is the most prominent and is occupied by the oral sucker. Between it and the two lateral lobes, which are slightly further back, are the pseudosuckers one on either side. The ventral fold extends as far forwards as the

lateral lobes. The cuticle covering the anterior half of the body has the same appearance as in the *Diplostomulum* stage. In optical section it appears to be armed with spines which do not penetrate the surface (Text-fig. 4).



Text-fig. 5. *Diplostomum pelmatoides* Dubois, ventral view showing part of the musculature and the general arrangement of the reproductive organs excluding the yolk glands.

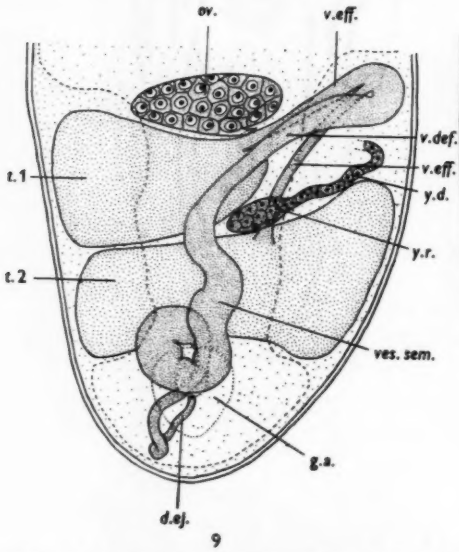
Text-fig. 6. Horizontal section showing longitudinal muscle bands in the parenchyma.

Text-fig. 7. Sagittal section showing opening of Laurer's canal.

Text-fig. 8. Sagittal section showing opening of hermaphrodite canal into the genital atrium.

The oral sucker is subterminal on the ventral surface and measures 0·050 mm. in length and 0·064 mm. in breadth. The ventral sucker is very slightly larger, measuring 0·055 × 0·070 mm. and lies just behind the middle of the anterior half of the body. The adhesive organ is situated close behind the ventral sucker and is usually covered partially or totally by the ventral body fold (Text-figs. 3–5). It is

circular in outline, measuring 0·100–0·106 mm. in diameter and opens by a median sagittal slit which as in the larva may give off rays laterally. Occasionally the organ may be evaginated. It is surrounded by gland cells embedded in the parenchyma which open into its cavity, or on to its surface in the evaginated condition; these are sometimes referred to as proteolytic glands. Diesing (1850) considered the opening of the adhesive organ to be the female genital pore. Brandes (1890) ascribed to the apparatus the role of digestion as he recognized the irritant, corrosive or even histolytic action of the secretion from the glands. Recent work by La Rue (1927*b*) van Haitsma (1931) and Baer (1933) confirms this interpretation and recognizes a chemical function for the adhesive organ. According to these authors the anterior



Text-fig. 9. *Diplostomum pelmatoides* Dubois. Male genitalia, ventral view.

Text-fig. 10. Female genitalia, ventral view. (Compare with Text-fig. 5; note sexual amphitypy.)

segment itself, owing to its deeply excavated form, and with the assistance of the oral sucker, pseudosuckers and ventral sucker, will assure the fixation of the parasite. The adhesive organ is thus brought into contact with the mucosa of the host where the secretion of its glands provokes histolysis of the host's tissues. The 'dissolved' material then passes forwards and is absorbed by the mouth. The pseudosuckers are complex apparently muscular organs forming two lateral more or less deep depressions situated one on either side of the oral sucker.

The musculature of the body wall is of the usual trematode type consisting of outer circular and inner longitudinal muscles. These are best developed in the anterior part of the body around the oral sucker and in relation to the pseudosuckers (Text-fig. 5). The deep longitudinal musculature is very well developed and consists of powerful longitudinal bands attached to the pseudosuckers on either side and extending backwards through the body (Text-figs. 5, 6). In whole mounts

they can be traced in the anterior segment of the body (Text-fig. 5), their position varying slightly according to whether the body is expanded or contracted. Horizontal sections are necessary to follow these fibres with accuracy into the posterior segment. It is found that they extend almost the whole length of the body (Text-fig. 6), some becoming inserted eventually into the body wall mainly dorsally but occasionally ventrally and others into the muscular genital atrium. The fact that most of them are inserted into the dorsal body wall may account for the concavity of the dorsal side in contracted specimens. Six or seven bands of muscle fibres occur on either side and as they pass backwards a certain amount of anastomosis occurs between neighbouring bundles.

The mouth in the centre of the oral sucker leads through a very short prepharynx into an elliptical pharynx measuring 0·048–0·052 mm. in length and 0·041–0·043 mm. in breadth (Text-figs. 4, 5). The oesophagus is so short as to be almost non-existent, bifurcating almost immediately into two caeca. The caeca diverge rapidly and pass on either side of the ventral sucker and adhesive organ to extend into the posterior segment of the body where they lie towards the dorsal side (Text-figs. 5–8). They are extremely difficult to follow into the posterior segment, due to the presence of the reproductive organs which fill this region.

As already mentioned the genital atrium, into which the male and female ducts open, is situated at the posterior extremity opening subterminally on the dorsal surface (Text-figs. 3, 9, 10). It is a deep cavity surrounded by a special arrangement of muscles, giving it the appearance of a sucker but is not as well defined or as clearly demarcated from the surrounding tissues as a sucker would be.

All the reproductive organs, with the exception of some of the yolk follicles, are situated in the posterior segment and it is interesting to note that sexual amphitypy is extremely common in this species.

The two large testes occupy the anterior two-thirds of the posterior segment of the body, being situated one behind the other (Text-figs. 3, 4, 9 and 10). The anterior testis is asymmetrical, being broader on the side nearest the margin of the body and narrowing towards the inner side (Text-figs. 9, 10). It measures 0·125–0·166 mm. in width and 0·083–0·125 mm. in length. This testis does not extend across the whole breadth of the body; it lies either to the right or to the left side. Of twenty-four specimens examined the anterior testis was on the right side (Text-fig. 9) in twelve and on the left side (Text-fig. 5) in twelve. This, of course, affects the position of the other reproductive organs in this region, giving rise to the amphitypy already mentioned (Text-figs. 5, 9). The posterior testis lying immediately behind the anterior occupies almost the whole breadth of the body (Text-figs. 9, 10). It measures 0·291 mm. wide and 0·200 mm. long; it is bilobed and concave ventrally (Text-fig. 3). The vas efferens from the anterior testis arises from the inner anterior margin (Fig. 9) and that from the posterior testis from the anterior margin to one side of the middle line. Both run forwards, obliquely at the side of the anterior testis as far as the junction between the anterior and posterior segments of the body. Here they unite to form the vas deferens which turns back enlarging to form a vesicula seminalis which passes to the middle line, ventral to the testes and dorsal to the uterus, and continues posteriorly enlarging gradually as it

goes. Behind the posterior testis the vesicula seminalis describes a complete turn towards the dorsal side. It then narrows quite suddenly to form the ductus ejaculatorius which passes back beyond the genital atrium almost to the posterior end of the body when it turns forwards again and joins the uterus to form a short hermaphrodite canal which opens into the dorsal side of the genital atrium (Text-figs. 3, 8). There is no cirrus or cirrus sac and no prostate glands have been observed.

The ovary is transversely oval in outline and is situated towards the dorsal side a little to the right (or left) of the middle line immediately in front of the anterior testis and at the junction of the anterior and posterior parts of the body (Text-figs. 3, 5, 9 and 10). It measures 0·087–0·117 mm. in transverse diameter and 0·041–0·050 mm. antero-posteriorly. The oocytes are large, measuring about 0·01–0·012 mm., and possess a hyaline nucleus 0·007 mm. in diameter with a conspicuous nucleolus. The oviduct arises from the inner posterior margin of the ovary (Text-fig. 10). It is a wide tube which proceeds towards the side of the body not occupied by the anterior testis. A short distance beyond its origin arises Laurer's canal which turns back towards the centre of the body and opens in the mid-dorsal line close behind the ovary (Text-figs. 7, 10). The oviduct then receives the yolk duct and after enlarging slightly to form the ootype continues laterally towards the margin of the body (Text-fig. 10). Here it turns ventrally and runs obliquely towards the middle line where it turns backwards taking a straight course to the genital atrium. It lies ventral to the vesicula seminalis and dorsal to the yolk glands (Text-figs. 3, 5). At its posterior extremity it is joined by the ductus ejaculatorius to form a short hermaphrodite canal which opens into the genital atrium (Text-fig. 8). The oviduct and uterus are lined with large cells containing conspicuous nuclei. Sperms have been found in the oviduct between its origin and Laurer's canal (Text-fig. 10).

The yolk glands are present in both segments of the body (Text-fig. 4). In the posterior segment they form a median band running along the centre of the ventral side, occupying about one-third of the width of the body. Behind the posterior testis they extend laterally on either side, occupying the whole depth of the body in this region. The yolk glands continue without interruption into the anterior segment where they occupy the whole width of the body. They extend forwards to just in front of the ventral sucker and almost to the bifurcation of the gut. The yolk reservoir is large and lies in the posterior segment of the body a little to one side of the middle line and behind the anterior testis (Text-figs. 5, 9 and 10). A short duct enters it ventrally (Text-fig. 3) from the yolk glands. The main yolk duct arises laterally (Text-figs. 5, 9 and 10), runs parallel to the early part of the uterus, turns upon itself and opens into the oviduct a short distance after the origin of Laurer's canal (Text-fig. 10).

The eggs are very large for so small a worm, measuring 0·087–0·093 × 0·048–0·062 mm. They are few in number, only from one to four being found in each adult worm.

These specimens compare very well with the description given by Dubois (1932). The measurements are very similar, the slight variations being due probably to varying degrees of pressure during fixation of the worms.

Other species of *Diplostomum* from *Mergus merganser merganser* are *Diplostomum mergi* (Dubois), *D. parviventosum* Dubois, and *D. pusillum* (Dubois). The last-named is much bigger than *D. pelmatoides* Dubois. *D. mergi*, also, is larger, and there is a very well-marked constriction between the two regions of the body. The shape of the anterior extremity is different and the ventral sucker and adhesive organ are separated from one another by some little distance. The nearest, in many respects, is *D. pusillum*, as this also is very small and the constriction between the two parts of the body is only feebly marked. The morphology, however, is different. The posterior segment is broader than long, the ventral sucker is in the middle of the anterior segment, the adhesive organ is large and usually overlaps the ventral sucker, and the yolk glands only reach as far forwards as the centre of the ventral sucker.

As already noted, the natural host of the worm in this country is not known, but it is hoped to find it in the near future, and also the early stages in the molluscan host.

SUMMARY

1. Large numbers of minnows (*Phoxinus phoxinus* (L.)) from Fron Goch Pool, near Aberystwyth, have been found to be heavily infested with *Diplostomulum pelmatoides* Dubois (= *D. phoxini* (Faust)), the parasites occurring in very large numbers in the brain, spinal cord and eyes.

2. The excretory system of the worm has been worked out in detail. There are fifty-two pairs of flame cells.

3. Infested heads, brains and eyes have been fed to the domestic duck and the adult stage of the worm *Diplostomum pelmatoides* Dubois obtained.

4. Some morphological details of the adult worm are described.

5. The natural bird host of the parasite in this country is not yet known, nor the stages in the molluscan host.

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KEY TO LETTERING OF FIGURES

(All drawings are semi-diagrammatic)

a.l.v.	antero-lateral vessel	*ov.*	ovary
a.p.t.	anterior primary collecting tubule	*ovd.*	oviduct
a.t.c.	anterior transverse commissural vessel	*p.l.v.*	postero-lateral vessel
		p.p.t.	posterior primary collecting tubule
c.c.	calcareous corpuscles	*p.t.c.*	posterior transverse commissural vessel
c.c.p.	common collecting trunk of primary system	*ph.*	pharynx
d.ej.	ductus ejaculatorius	*pph.*	prepharynx
d.l.m.	deep longitudinal muscles	*ps.*	pseudosucker
e.	egg	*s.c.m.*	superficial circular muscles
e.p.	excretory pore	*s.l.m.*	superficial longitudinal muscles
e.v.	excretory vesicle	*t.* 1 & 2	testes 1 and 2
g.a.	genital atrium	*ut.*	uterus
h.c.	hermaphrodite canal	*v.b.f.*	ventral body fold
hf.	hold-fast organ	*v.def.*	vas deferens
int.	intestine	*v.eff.*	vas efferens
l.c.	Laurer's canal	*ves.sem.*	vesicula seminalis
l.c.v.	lateral collecting vessel	*y.d.*	yolk duct
m.d.v.	median dorsal vessel	*y.g.*	yolk gland
oot.	ootype	*y.r.*	yolk reservoir

EXPLANATION OF PLATE V

Photomicrographs by J. E. Welbourn

Fig. 1. Sagittal section through the head of a minnow showing a normal brain.
Fig. 2. Sagittal section through the head of a minnow showing the brain infested with *Diplostomulum pelmatoides* (*D. phoxini*). This fish showed no symptoms.
Fig. 3. Sagittal section through a very heavily infested brain. This fish showed symptoms of distress.
Fig. 4. Transverse section through the brain of a minnow showing diplostomula in the optic vesicles and aqueductus sylvii.
Fig. 5. Transverse section through the anterior end of the spinal cord of a minnow showing diplostomula and vacuolated tissue.
Fig. 6. Transverse section through the spinal cord of a minnow showing diplostomula near the surface and deeply embedded in the cord.

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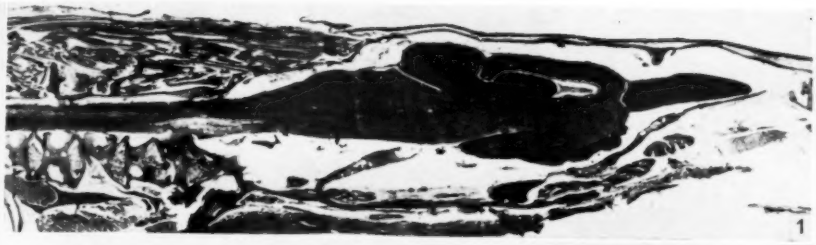
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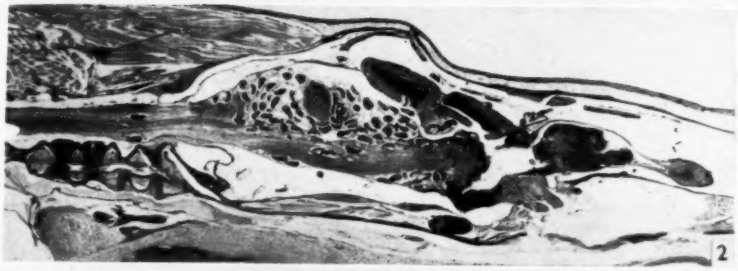
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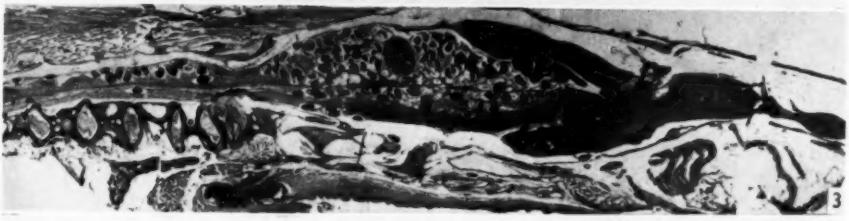
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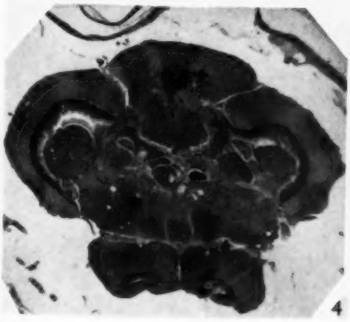
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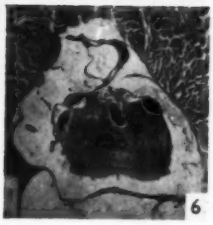
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THE LIFE HISTORY AND BIONOMICS OF *DICLIDOPHORA DENTICULATA* (TREMATODA: MONOGENEA)

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(With 21 figures in the text)

CONTENTS

	PAGE
I. Introduction	314
II. Material and methods	314
The host	314
Detection and removal of flukes	316
Observation of living flukes	317
Culture of eggs	317
Preparation of hosts and their experimental infestation with larvae	317
Recovery of larvae from experimental infestations	317
Fixation and staining of adults and larvae	317
Fixation and staining of embryos	318
Nomenclature used	318
III. Observations	319
The egg	319
Oviposition	319
Maturation of the gametes and fertilization	320
Embryology	320
Fate of yolk	322
Hatching	322
The ciliated larva	322
Larval development	325
Rate of development and longevity	328
Reproduction	331
Effect of salinity variation	331
Position and movement	332
Degree of infestation and distribution on gills	333
Pathogenicity	334
Host specificity and immunity	334
Geographical distribution	336
IV. Discussion	336
The egg and oviposition	336
Maturation of the gametes and fertilization	336
Embryology	337
Fate of yolk	338
Hatching	338
The ciliated larva	338
Larval development	341
Rate of development and longevity	342
Reproduction	344
Effect of salinity variation	345
Position and movement	345
Degree of infestation and distribution on gills	346
Pathogenicity	346
Host specificity and immunity	346
Geographical distribution	348
V. Summary	348
References	349

I. INTRODUCTION

The overriding economic importance of digenetic trematodes due to their pathogenic activities has led to a great deal of research being devoted to their study. Monogenetic trematodes have no bearing on the field of applied science except in so far as some of them cause epidemics in fish hatcheries and, as a result, most of the work which has been done on them has been confined to the simple description of species with no attempt to determine their biology or even their life cycles. However, some of the free-swimming larvae have been described and, in a handful of cases, the whole life history has been worked out. The present study adds one species, *Diclidophora denticulata*, to this short list, and an attempt has also been made to learn something of its biology.

The species, *D. denticulata*, was first described in 1875 by Olsson under the name of *Octobothrium denticulatum*. His account, though sufficient to identify the animal and distinguish it from related species, was incomplete and contained some errors. Later, Cerfontaine (1896) studied the fluke in detail, amplifying and correcting Olsson's account. Since then, this worm has not specifically been the subject of any further study, although Price (1943) gave a diagnosis of it.

The work here described was carried out during tenure of a post-graduate scholarship for which I am indebted to the University of St Andrews. It is a pleasure to record my thanks to Prof. H. G. Callan, in whose Department the work was done, and to Mr D. R. R. Burt, also of the Department of Natural History, St Andrews, for his advice and help. I am grateful to Dr J. Llewellyn for kindly allowing me to read the manuscript of his paper on the food and gut pigment of the Polyopisthocotylea before publication, and also to Mr D. C. W. Smith, Dr J. Llewellyn and Miss E. M. Shelswell for permission to use the unpublished material referred to in the text.

II. MATERIAL AND METHODS

The host

The flukes were obtained from the gills of *Gadus virens*. The fishes were nearly all taken from St Andrews Bay, Fife, being caught with hook and line, normally from a dinghy, over rocks. A few were obtained through the kindness of other fishermen in the neighbourhood and, of these, a small number were caught in the Firth of Forth. The length of each fish and the date on which it was killed were recorded. In a few cases, where only the heads of the fishes were available, their total lengths were estimated by using a formula devised by Smith (unpublished work). He found that the relationship between the length of the head (H) and the total length (L) in *G. virens* is

$$H/L = 0{\cdot}2405,$$

with a possible error in estimating the total length from the head length of $\pm 0{\cdot}4$ cm. This error can be ignored. There is a correlation between this ratio and total length such that there is an increase in relative size of the head with the size of the fish. Where, as here, the fishes measured do not vary greatly in size, this again can be ignored. Total length was, accordingly, estimated from the formula

$$\text{Total length} = \frac{\text{length of head}}{0{\cdot}2405}.$$

Bertelsen (1942) said that *G. virens* began to spawn off the Scottish Coast in January and that the fry were pelagic for the first 3 months of their life. This is in accordance with Smith's (unpublished) finding that the fry began to appear

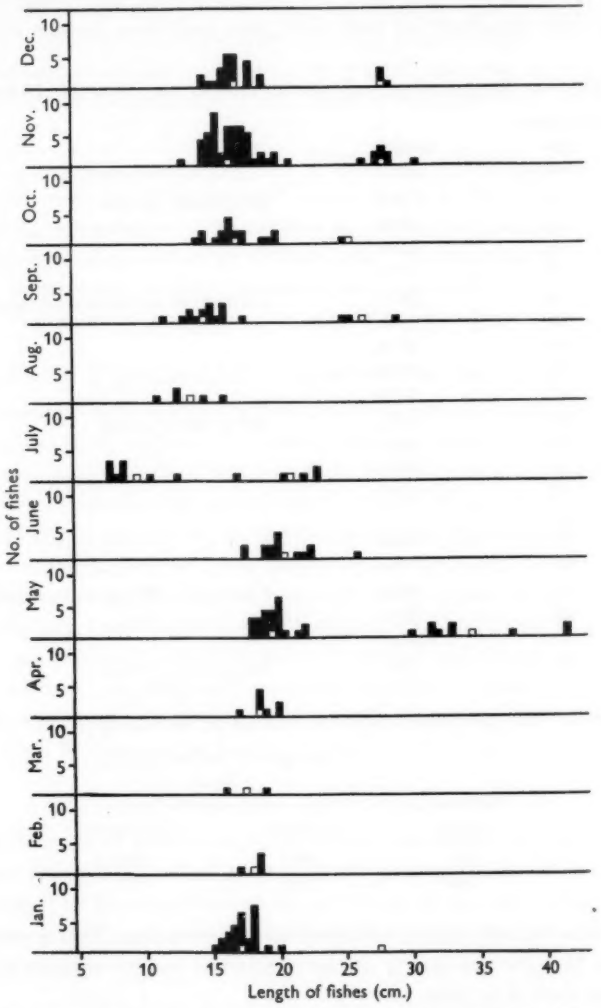

Fig. 1. Size frequency per month of *Gadus virens* at St Andrews. Average lengths white, outlined black.

in rock pools at St Andrews in April. The lengths of the fishes caught in St Andrews Bay and neighbourhood and during the present investigation are shown in Fig. 1. From the composite picture thus built up, the average lengths in each natural group give the estimated growth rate of an individual fish. This is shown in Table 1. A study of the scales of these fishes showed them to belong to the first two growth periods, and the sizes attained at the end of each period are shown in

Table 2. Numbers examined were insufficient to give the maximum size reached by fishes during the second growth period. These results accord with those derived from Fig. 1.

Bertelsen (1942) found that, after the pelagic period, the young fishes came inshore where they remained for their first year, and then, except for a few which

Table 1. *Estimated growth rate of* Gadus virens *at St Andrews*

Estimated age (months)	Length (cm.)	Growth period
5	9·0	1st growth period
6	13·5	
7	14·5	
8	17·0	
9	16·5	Formation of 1st annual ring
10	17·0	
11	17·0	
12	18·0	
13	17·5	
14	18·5	2nd growth period
15	19·5	
16	20·5	
17	21·0	
18	—	
19	26·5	
20	25·5	
21	28·0	Formation of 2nd annual ring
22	28·0	
23	27·5	
27	34·5	3rd growth period

Table 2. *Size attained by* Gadus virens *at the end of growth periods*

Growth period	Length at end of period (cm.)	
	Average	Maximum
1st	16·5	19·5
2nd	27·5	?

remained in the littoral region, returned to deeper water. This would account for the fact that the great majority of the specimens caught inshore at St Andrews were fishes in their first year.

Detection and removal of flukes

Fishes for examination were killed and the gills removed immediately to Petri dishes of sea water. The lamellae were scanned under low-power binoculars and the flukes removed with blunt needles and a pipette, or, for sectioning of flukes *in situ*, the lamella bearing the fluke was cut off. Records were kept of the developmental stage of every fluke and from which pair of gills it had been taken.

Observation of living flukes

The general behaviour of living flukes was studied in sea water with low-power, dissecting binoculars, but more detailed observations were made under higher power, the worms being suspended in hanging drops or lightly compressed under a coverslip. To reveal the excretory system, larvae were strongly compressed under a coverslip. Experiments were carried out to test the response of larvae to various stimuli; details of the methods used are included in the account of larval behaviour.

Culture of eggs

Eggs were set aside for development in sea water in small, deep Petri dishes which, to prevent evaporation, were put in a larger covered vessel partly filled with water, and kept in a temperature-controlled tank. The sea water was renewed from time to time. Sometimes Berkfeld-filtered water was used and, although this was not essential, it helped to keep down the growth of moulds on the egg shells.

Preparation of hosts and their experimental infestation with larvae

Fishes required for experimental infestation were first deparasitized by a modification of Alvey's (1936) technique: 2 parts of a sea-water solution of chloretone (trichlor-tertiary butyl alcohol), saturated at room temperature, were added to 5 parts of sea water and the fish left in this for 5 min. This led to total anaesthesia of the fish, and all, or almost all, of the flukes present died and dropped off. The fish was then revived in pure sea water, the process being aided by causing a current of water to flow over the gills. One or two days later, experimental infestation was carried out. The fish, together with a Petri dish containing larvae of 3–4 hr. of age, was placed in sea water in a small aquarium provided with an aerator. After 4 hr., the fish was removed to permanent aquarium quarters. Infestations failed when periods in the small aquarium were much longer than 4 hr., perhaps because the larvae were killed by the accumulation of waste products in so small a volume of water.

Recovery of larvae from experimental infestations

At varying lengths of time after exposure to free-swimming larvae, the fishes were killed and the gills examined to recover the larvae and determine their degree of development. The finding of a number of larvae of the same stage of development was taken as being satisfactory evidence that these were the larvae supplied to the fish experimentally.

Fixation and staining of adults and larvae

Owing to the contractility of the fluke, it is difficult to fix specimens so as to give reliable measurements of size and proportions. The most constant results were given by leaving the worms in 20 % magnesium sulphate solution until they did not move when touched or shaken. They were then laid on a slide, if necessary under very gentle pressure from a coverslip, and fixed. Free-swimming larvae for all purposes were pipetted suddenly into a large volume of fixative. Fixatives used included Bouin's fluid, Schaudinn's fluid made up in sea water, and Carnoy-

Lebrun's fixative, but the most satisfactory, causing least shrinkage of tissue and artificial coalescence of shell globules, was chromo-nitric acid (Gatenby & Painter, 1937). For whole mounts the worms were usually stained with Gower's (1939) modified carmine, occasionally with light green as a counterstain. Paraffin-embedded sections were cut at various thicknesses from 6 to 15μ and stained either with Heidenhain's haematoxylin and eosin or Heidenhain's azan stain (Pantin, 1946).

Fixation and staining of embryos

Great difficulty is always experienced in achieving satisfactory fixation of embryos which are enclosed in impermeable egg shells, and *D. denticulata* is no exception. Immersion of the eggs in a hypertonic solution led to collapse of the shells and crushing of the contents. Attempts to puncture the shell mechanically were unsuccessful owing to its smoothness and resilience. The following method of fixation and subsequent opening of the shell by chemical means was eventually worked out and gave tolerably satisfactory results.

Acetic sublimate (Halkin, 1901) was added to the eggs, a few drops at a time, as they lay in sea water. They were then left in the full strength fixative for some hours. After washing in water, excess sublimate was removed by leaving them for a while in a very dilute aqueous admixture of Lugol's solution (Pantin, 1946). After a further wash in water they were transferred very gradually to 25 % aqueous solution of eau de Javelle (Halkin, 1901) and kept under observation. When the filaments had largely dissolved away and the shell seemed to be on the point of rupture, usually after about three-quarters of an hour, the eggs were transferred suddenly to distilled water. This, by endosmosis, ruptured the shell along the opercular line. Immediately this had occurred, the eggs were transferred to Belling's carmine (Gatenby & Painter, 1937), thus preventing further swelling and disruption of the egg contents which rapidly occurred in water. Eggs required for whole mounts were dehydrated, cleared in clove oil and mounted in balsam, the carmine being the only stain. Chatton's technique (Langeron, 1925) was used in sectioning eggs. Most of the carmine was lost during the processes and the sections were restained on the slide with Heidenhain's haematoxylin and eosin.

Nomenclature used

The names of Monogenea adopted are those considered correct by Sproston (1946), and these have been used throughout regardless of the fact that authors to whom reference is made have sometimes used other names. In order to avoid confusion, a list* of these synonyms is given here:

Benedenia melleni (MacCallum, 1927) Johnston, 1929.

As *Epibdella melleni* by Jahn & Kuhn (1932), Nigrelli & Breder (1934) and Nigrelli (1937).

Diclidophora denticulata (Olsson, 1875) Price, 1943.

As *Octobothrium denticulatum* by Olsson (1875) and Braun (1893).

As *Dactylocotyle denticulatum* by Cerfontaine (1896, 1898).

* Full references are given by Sproston (1946).

As *Dactylocotyle denticulata* by Baylis & Jones (1933) and Rees & Llewellyn (1941).

Diclidophora luscae (van Beneden & Hesse, 1863) Price, 1943.

As *Dactylocotyle luscae* by Gallien (1934).

Diclidophora palmata (Leuckart, 1830) Diesing, 1850.

As *Dactylocotyle palmatum* by Cerfontaine (1898).

Diclidophora pollachii (van Beneden & Hesse, 1863) Price, 1943.

As *Dactylocotyle pollachii* by Gallien (1934).

Diplorchis scaphiopodis Rodgers, 1941; emend. Sproston, 1946.

As *Diplorchis scaphiopi* by Rodgers (1941).

Kuhnia scombri (Kuhn, 1829) Sproston, 1945.

As *Octobothrium scombri* by Gallien & Le Calvez (1947).

Neodactylogyrus macracanthus (Wegener, 1909) Price, 1938.

As *Dactylogyrus macracanthus* by Wilde (1936) and Gröben (1940).

Polystoma integerrimum (Fröhlich, 1791) Rudolphi, 1808.

As *Polystomum integerrimum* by Gallien (1935).

Polystoma nearcticum (Paul, 1935) Price, 1939.

As *Polystoma integerrimum nearcticum* by Paul (1938).

III. OBSERVATIONS

The egg

In form the egg (Fig. 2) of *D. denticulata* is constant, being elliptical, tapering slightly more abruptly toward the posterior than the anterior end. The size is slightly variable, the egg proper measuring $191{\cdot}5 \pm 6{\cdot}5\mu$ in length by $78{\cdot}8 \pm 2{\cdot}0\mu$ in breadth. Beyond this, terminal filaments project. These are intermittently hollow. The anterior, measuring about 240μ in length, ends in a crook. The posterior filament is considerably longer, having a length of about 800μ and its extremity is expanded into a crenulated disk with a central depression and nine marginal projections. The shell is tough and resilient, translucent and, when mature, golden brown in colour. Its anterior part forms an operculum separated from the rest of the shell by an invisible line of weakness running slightly obliquely as indicated by the dotted line in Fig. 2. Even new-laid eggs may rupture along this line if roughly handled. The contents of this ectolecithal egg consists of a mass of yolk cells surrounding an ovum which lies in a rather anterior position.

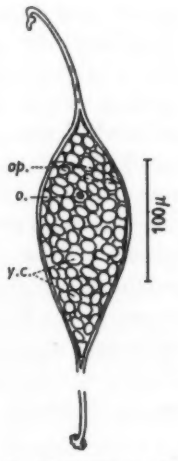

Fig. 2. The egg. *o.*, ovum; *op.*, opercular line; *y.c.*, yolk cells.

Oviposition

The eggs are laid in sheaves of 100 to 200 together with the posterior filaments still entwined. Oviposition is brought about by powerful contractions of the body, the head region being drawn back. The anterior

crooks evidently do not serve the function of anchoring the eggs on the gill lamellae of the parent's host since, although new-laid eggs were sometimes found in the gill mucus of freshly killed fishes, developing eggs were never found attached to any part of the host.

Maturation of the gametes and fertilization

The small size of the chromosomes makes a study of cell division very difficult and nothing was determined concerning the maturation of the spermatozoon, which must merely be presumed to contain the haploid number of chromosomes on penetrating the oocyte. It is occasionally seen as a small, lobular pronucleus lying to the side of the ovum in mature or new-laid eggs, but it was not possible to detect when or where fertilization took place.

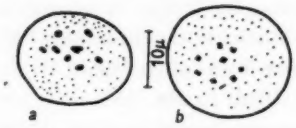

Fig. 3. Maturation of the oocyte. *a*, first meiotic metaphase; *b*, second meiotic metaphase.

A little more evidence was gathered concerning the ovum. Polar bodies were never seen, perhaps because they soon disintegrate, and the ovum is in any case surrounded by yolk cells, but maturation can be inferred from the number of chromosomes seen in sections of early stages of development. Thus, metaphase stages are seen during the first 2 hr. of development, before cleavage has begun, in which there are nine chromosomes (Fig. 3). Some of these may represent nine bivalents (*a*), but others show the separation of the chromatids characteristic of the second meiotic metaphase (*b*). Similarly, anaphase and telophase stages are found in which nine chromosomes are passing at least to one pole, though it cannot be said with certainty, owing to technical difficulties, that nine chromosomes pass to each pole. In a few of these telophase stages nine chromosomes are seen projecting in a small cytoplasmic bulge from the main part of the ovum. Other one-celled stages show divisions in which more than nine chromosomes are involved. Approximately eighteen could be counted but their exact number was difficult to make out owing to their small size and crowded arrangement. In prophase the chromosomes appear as delicate, thread-like bodies, which condense into short, thick rods, or oval forms, in metaphase. Some of those seen in anaphase and telophase are V-shaped.

Embryology

Cleavage begins at a varying time during the first 5 hr. after laying as is shown in Fig. 4. It is holoblastic and somewhat unequal (Fig. 5), leading to the formation of an irregular morula in which no germ layers can be distinguished. There is no evidence of spiral cleavage. The interphase nuclei of cleavage stages differ from the

primary oocyte in having a lobular border and a number of nucleoli. The first few cleavage divisions follow one another relatively rapidly so that four-celled stages are common a few hours after laying, but after 24 hr. the majority of the eggs have only reached a six-celled stage. At 48 hr. the embryo is a roughly spherical morula of about twelve cells. During the next 2 or 3 days the embryo enlarges and takes on an oval shape: occasionally a slight central cavity can be seen. The cells composing the embryo are similar in appearance except that some have nuclei which, in the fixed condition, are clear whereas others are granular. These two types are mixed indiscriminately.

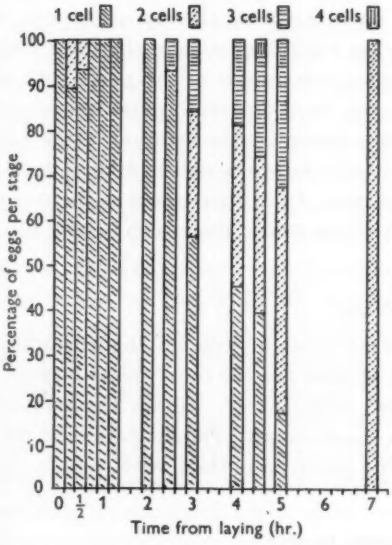

Fig. 4. Condition of the eggs during the 7 hr. following laying.

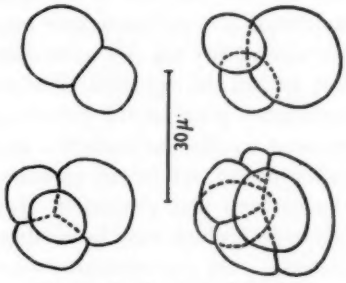

Fig. 5. 2-, 3-, 4- and 6-celled embryonic stages.

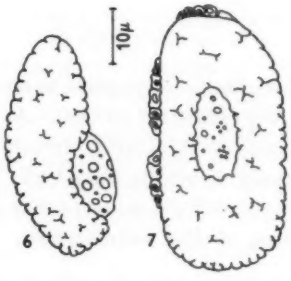

Fig. 6. 6-day embryo.
Fig. 7. 11-day embryo.

Six days after the laying of the egg, the peripheral cells of the embryo have been modified to form an epithelium. The general shape of the embryo is a slightly flattened ovoid with a depression in one face filled with adherent yolk (Fig. 6). Later, this depression is found to be ventral. Further growth results in the yolk in the depression being overgrown by the surrounding cells and incorporated in the embryo (Fig. 7). The cells of the mouth and pharynx are differentiated at about the time this epiboly is complete.

After 12 days of development, the epithelium is shortly ciliated in those areas where there are cilia in the hatched larva. Four pairs of lateral, larval hooks and a pair of postero-lateral hooks behind the foregoing are being laid down in the posterior, haptorial part of the embryo. One day later the development of these

hooks is completed and the stout, hindmost, median pair of hooks is forming. The mouth is clearly differentiated, and from the pharynx a narrow canal can be seen running to the sac of enclosed yolk, or larval gut sac. Refinement of the larval form follows and, with further growth, the embryo is only accommodated in the available space by being bent into an S-shape.

Fate of yolk

The yolk cells do not break down immediately the egg is formed, but persist through the first day of development as discrete units which show, in the fixed condition, a mono-nucleolate nucleus and cytoplasm containing fine granules. On the second day the cell walls disintegrate and nuclei and cytoplasm are seen lying free within the egg shell. Further disintegration and reduction in volume of the yolk takes place as the embryo grows, some being, as already mentioned, incorporated within the embryo. In the living condition, during the later stages of development, refringent globules of varying size appear in the yolk, both within the embryo and around it. Inspection of fixed material shows corresponding 'vacuoles' which may be assumed to have arisen by the solution of the globular material. On one occasion two eggs, out of a bunch which had been developing for 3 days, were found to contain no embryos. In neither case was there any sign of disintegration of the yolk cells which still resembled those seen in new-laid eggs.

Hatching

For about 3 days prior to hatching the larva can be seen moving within the shell and the cilia start to beat. As hatching approaches the movements become more vigorous and the larva stretches and turns convulsively. Finally the operculum is partially or wholly forced off. The larva squeezes out, remains poised on the edge of the shell for a moment, the cilia moving gently, and then, with sudden vigorous beating of cilia, swims away.

The ciliated larva

The ciliated larva (L^0) (Fig. 8), which emerges from the egg is elliptical and flattened dorso-ventrally. The anterior end is blunt and from here the body widens somewhat, and then tapers to a rounded posterior end with a median cleft from which projects a small, clear vesicle. From the ventral surface of the posterior quarter of the body, but not reaching the hind-end, there arises a haptor (*h.*) expanded laterally into two wings, each bearing four hooks along their lateral margins (*l.h.*). Posterior and median to these lateral hooks are two postero-lateral hooks (*p.l.h.*) and, between these, a pair of large, median hooks (*m.h.*). Though very contractile, at average extension the animal, when fixed, is $138{\cdot}0 \pm 3{\cdot}5\mu$ long and $59{\cdot}0 \pm 12\mu$ across at the broadest part. The lateral and postero-lateral hooks are the same shape, having a long, very slightly curved shaft, a short, ventral, backwardly directed guard and a gently curved blade: they are $20{\cdot}0 \pm < 0{\cdot}5\mu$ long. The median hooks, $15{\cdot}0 \pm < 0{\cdot}5\mu$ long, differ in having a straight shaft, no guard and a much more robust, strongly recurved blade.

Over the hind-part of the body behind the haptor, the epithelium is thickened and ciliated (*c.e.*). Anteriorly to the haptor a strip of ciliated epithelium runs forward, confined to the lateral margin of the body except for a slight expansion on to the ventral surface just in front of the haptor. Finally, it leaves the lateral margin to meet dorsally its fellow from the other side about 10μ behind the anterior tip of the body. The cilia are 11μ long.

The mouth (*m.*) is terminal and large with tumid, suctorial lips. It leads, via the prepharynx, to the pharynx (*p.*). The position of the latter is in life very variable, so that at one minute there appears to be no prepharynx and at the next it is of considerable extent. Since the wall of the prepharynx makes contact with the posterior margin of the pharynx, the latter organ lies, as it were, within the former. The pharynx itself is a spherical body with a transverse diameter in the fixed state of $16{\cdot}5 \pm 1{\cdot}5\mu$. It has thick muscular walls and a very restricted internal cavity whose axis is inclined backwards and obliquely dorsally to that of the body. From the pharynx there extends a restricted passage which widens out into the gut sac (*g*). This narrow cavity is, like the prepharynx, of variable length according to the position of the pharynx, and sometimes the pharynx lies close to the gut sac. This last is a wide cavity of indeterminate and variable shape, sometimes extending into the haptor almost as far as the roots of the median hooks. It contains residual yolk with globules and a few, refringent, yellowish aggregates thought to be shell material. Also arising from the hind-end of the pharynx is a pair of laterally and postero-dorsally directed blind diverticula (*g.d.*) which reach to the level of the anterior margin of the gut sac. There is no differentiation of the cells surrounding the alimentary ducts apart from the muscular nature of the pharyngeal wall. Tissue of a different nature from the remaining parenchyma, and staining deeply in the vital stain, brilliant cresyl blue, extends in two lateral fields between the mouth and the anterior margin of the gut sac. Refringent spheres of varying size are seen here and there in the body.

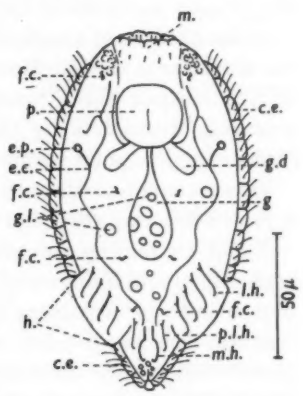

Fig. 8. Semi-diagrammatic ventral view of ciliated larva. *c.e.*, ciliated epithelium; *e.c.*, excretory canal; *e.p.*, excretory pore; *f.c.*, flame cell; *g.*, gut sac.; *g.d.*, gut diverticula; *g.l.*, globules; *h.*, haptor; *l.h.*, lateral hook; *m.*, mouth; *m.h.*, median hook; *p.*, pharynx; *p.l.h.*, postero-lateral hook.

The excretory system already shows the basic pattern of that seen in the adult. There are four pairs of flame cells (*f.c.*): the first pair antero-lateral to the pharynx, the second pair antero-lateral to the gut sac, the third pair postero-lateral to the gut sac and the fourth pair external to the roots of the median hooks. Apparently single, longitudinal, collecting tubules lie one on each side of the body (*e.c.*). At about the level of the fore-end of the gut sac, from each longitudinal canal, a short duct is given off which runs forward and outward to open in a dorso-lateral excretory pore (*e.p.*).

No nervous system or sensory organs could be detected and the reproductive system is not yet differentiated.

The newly hatched larva is very active and changeable in shape. It swims vigorously by the action of the posteriorly beating cilia, either straight forward or spiralling on its own axis. Sharp turns are frequently made, the dorsal surface being directed toward the inner side of the arc. The animal moves up and down while whirling round in a right-handed spiral. Frequently it comes to rest and sinks slowly until it darts off again but, if on the bottom or supported by the surface film, it may move about by alternate contraction and elongation of the body.

In the absence of a suitable host this phase persists for about 12 hr. after which the movements become more lethargic, the larva sinks to the bottom and sloughs off the ciliated coat. Specimens may be seen creeping over the substratum, attaching themselves by the suctorial mouth and attempting to grip with the hooks. Death occurs within 24 hr. of hatching if no host is found.

Various experiments were tried in an attempt to determine whether there were stimuli attracting the free-swimming larva to its host.

A dish containing a number of vigorous, ciliated larvae was placed on a black background and illuminated from the side by a powerful beam of light. The larvae were seen to be swimming about normally. A black shade was then arranged so that the dish was divided vertically into two sections, one, as before, powerfully illuminated and the other only lit by low intensity, diffuse daylight. The shade was left in place for 30 min. and the behaviour of the larvae noted. They could not be seen in the shaded zone but were swimming normally in the lit region and making their way in and out of the shaded zone without change of behaviour on encountering light of a different intensity. Some were observed making upward spirals, half of each turn being in the brightly lit zone. When the shade was removed, the concentration of larvae in each zone was immediately noted and there was found to be no preponderance of larvae in either region. Over a period of 2 min. a count was kept of the number of upward spirals made by a group of larvae in a dish on a dark ground and illuminated strongly from the side: twenty spirals were made. The upper half of the dish was then shaded and the spirals made in the following 2 min. noted: there were eighteen. A small piece of absolutely fresh gill of *Gadus virens* was put in a dish with a number of vigorously swimming larvae and their behaviour observed over a period of 1 hr. There was no concentration of larvae on or around the gill and those which encountered it swam on without any change of behaviour. After 3 hr. the gill was again examined, but no larvae had attached themselves to it. A similar experiment was carried out in which some of the larvae used were old and tiring. One or two of these, having reached the gill, attached themselves to it by the mouth and then embedded the hooks in the host tissue. With many contortions the ciliated coat was ripped off, the triangular, posterior part of the body being drawn out of the investing epithelium last. Any subsequent movements, made after the larva has settled on the host, are leech-like, the larva gripping first with the mouth and then with the haptor. In this way larvae move over the gills, but are usually seen clinging to the distal lamellae by the haptor.

Larval development

When the larva has settled on the gills of the host and discarded the ciliated coat, its subsequent development consists of growth and gradual acquisition of the adult characters. There is no sudden metamorphosis between the larval stages, which are here named separately purely for convenience and clarity.

Table 3. *Range of measurements in μ of* Diclidophora denticulata *during development*

Stage	Total length	Haptorial length	Breadth	Transverse pharyngeal diameter	Average transverse clamp diameter
L^0	138·0 ± 3·5	—	59·0 ± 12·0	16·5	—
L^1	125·0–133·0	—	53·0–61·0	—	—
L^2	276·5–308·5	—	69·5–85·0	29·5–37·0	53·0–55·0 (only pair)
L^3	312·0–574·0	—	89·0–158·5	34·5–45·0	58·5–69·0 (post. pair)
L^4	703·0	—	148·5	50·5	61·0 (post. pair)
L^5	772·0–831·5	208·0–247·5	168·5–178·0	56·0–	69·0– (post. pair)
I	1260–2975	368–1085	245–560	53·0–109·0	152·0–286·0 (1st 3 pairs)
A	2100–7105	630–2555	315–2345	77·0–151·5	232·5–688·0 (all pairs)

The first-stage larva (L^1) resembles the ciliated larva apart from the absence of the ciliated coat. Its measurements are given in Table 3, from which it is seen that larvae of this stage are smaller than ciliated larvae. At 3 days of age gland cells, staining deeply in azo-carmine, are seen lying near the pharynx and in a glandular pocket of the dorsal lip, which, in the adult, has been seen exuding a sticky, mucus-like secretion. The body tissue is largely composed of parenchyma, but a few, larger cells with mononucleolate nuclei occur in mid-body. The dorsal nerve commissure and the two major longitudinal nerves can be made out at this stage.

Between 5 and 13 days after hatching the second stage of development (L^2) is reached (Fig. 9). The hindmost pair of lateral hooks have been superseded by a pair of definitive clamps. These are formed externally and slightly dorsally to their corresponding, larval hooks, the latter disintegrating when their function is taken over by the clamps. The remaining hooks are fully functional. Though the path of the gut is difficult to follow, it is almost certainly still a median sac. Vitelline cells are already differentiated to the extent of showing a patchy staining reaction of the cytoplasm, but no other genital differentiation has taken place. Experimental infections show that this phase persists beyond 38 days. The range of measurements of second-stage larvae is given in Table 3. It is in larvae of the second stage that the membrane lining the gut is first seen to bear at irregular intervals the 'pigment cells' so characteristic of the adult. They contain numerous, fine, brown granules, and similar granules are seen lying free in the lumen of the gut. In some living

larvae and adults the gut lumen contained a pink fluid, and in a few larvae of this and later stages the gut was filled with elliptical to spherical cells having clear cytoplasm and deeply staining nuclei. They were of approximately the same dimensions, though not having quite the same appearance, as the host's red blood corpuscles, which could be examined alongside when the worm had been sectioned *in situ*.

Attempts to keep experimental fish for longer than 38 days after infection failed so it is not known when the third larval stage (L^3) is reached by the formation of the second pair of adult clamps. In worms of this stage collected from natural infections it was found that the second pair of clamps arises, like the first pair,

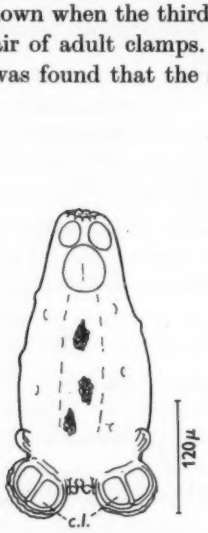

Fig. 9.

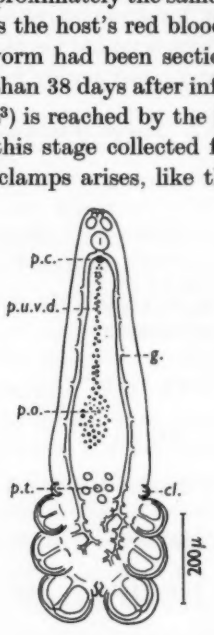

Fig. 10.

Fig. 9. Semi-diagrammatic ventral view of second-stage larva. *cl.*, 1st pair of clamps.

Fig. 10. Semi-diagrammatic ventral view of fifth-stage larva. *cl.*, 4th pair of clamps; *g.*, bifurcate gut; *p.c.*, primordium of cirrus; *p.o.*, primordium of ovary; *p.t.*, primordium of testes; *p.u.v.d.*, primordium of uterus and vas deferens.

externally to the corresponding pair of larval hooks, and that the median, postero-lateral and two anterior pairs of lateral hooks are still intact. The gut is now plainly bifurcate, rejoining and then branching in a palmate manner in the haptorial region. There is no change in the vitelline cells, but a mass of closely set cells with deeply staining, reticulate chromatin in their nuclei is differentiating in mid-body to form the genital primordium. This area is surrounded by a few large cells which have spherical nuclei showing, when fixed, one or two closely opposed nucleoli and a coarse, chromatic network. Their cytoplasm is finely granulated and stains sharply with aniline blue. The observed range of measurements of this stage is shown in Table 3.

The fourth larval stage (L^4) is attained with the formation of the third pair of clamps, this taking place before the second pair has grown to the size of the first pair. The median, postero-lateral, and most anterior pair of lateral hooks persist. The general condition of the larva is unchanged from the previous stage except that there is increased differentiation of the genital region. The mass of deeply staining cells in mid-body now extends as a narrow column along the future course

of the uterus and vas deferens; there may also be an expansion of this column at its anterior end. The large, blue staining cells now surround the genital region more and more extensively. Slight indications are present of cells differentiating as testicular primordia. Each of these small cells has a nucleus which, when fixed, is seen to have a small, central nucleolus from which radiates a delicate, chromatic network. Measurements of the fourth larval stage are given in Table 3.

The formation of the final and most anterior pair of clamps introduces the fifth larval stage (L^5) (Fig. 10). This last pair of clamps is formed, as in the case of the fourth larval stage, before the preceding pair has reached the size of the earlier formed pairs. The external teeth, characteristic of the adult, are now present on the more posterior clamps. All the lateral hooks have disappeared, being superseded by clamps, and, though the median hooks are intact, the postero-lateral ones are degenerating. The gut shows increasing posterior anastomosis and subsidiary branching. In a few cases the genital patch in mid-body is beginning to show slight differentiation into primordia of the ootype and the ovary and, at the anterior end of the genital column, a ring of regular, hook generating cells is sometimes seen in the position of the cirrus. A few late stages show cirrus hooks being laid down, one in each of these cells. The observed range of measurements of fifth-stage larvae is shown in Table 3.

When the fourth pair of clamps has reached the size of the more posterior pairs, an immature phase follows, during which growth of the whole animal continues and the elaboration of the reproductive organs is completed.

In the earliest immature adult stage the only advances in reproductive development are the presence of several cells in each testicular acinus and further development of the cirrus hooks. With the growth of the hooks, the cells secreting them become stretched along the backs of the hooks and finally disintegrate. By the time the cirrus hooks are fully formed, the branching of the gut has reached the degree of complexity seen in the adult and the pigmented cells along its walls are very numerous. The gland cells lateral to the pharynx are present, but now have the smaller proportions relative to body size which are characteristic of the adult. Large cells, as in earlier stages, surround the genital primordium in which two parts can now be distinguished: the ovarian mass and a solid body of cells representing the ootype. The column of cells leading anteriorly from this mass is now separated into two solid strips of cells destined to form the uterus and the vas deferens respectively. There is as yet no sign of the receptaculum seminis and the vitelline cells remain in the state reached during the second larval stage. A number of cells are seen in each testicular acinus, but none has divided to form morulae. The remaining larval hooks, the median and postero-lateral pairs, are degenerating.

With increased differentiation of the ovary in which young oocytes are formed, and of the testes where morulae of varying degrees of maturity appear, a change occurs in the vitellaria, so long quiescent. Each vitelline acinus now contains several cells still showing the typical, patchy cytoplasm of earlier stages. By a breakdown of the cells of the core, a central lumen is formed in the ootype, uterus and vas deferens. Finally, the receptaculum seminis is formed, but it has never

been seen containing spermatozoa in an animal which has not reached sexual maturity. Measurements of immature animals (I) are given in Table 3.

Maturity is attained by the growth and discarge of oocytes from the ovary, the filling of the vas deferens with ripe sperm, the elaboration of shell globules in the growing vitelline cells and the passage of the latter to the vitelline reservoir.

The relative bodily proportions during growth from the fifth larval stage onwards are expressed graphically in Figs. 11–13. Measurements of total length,

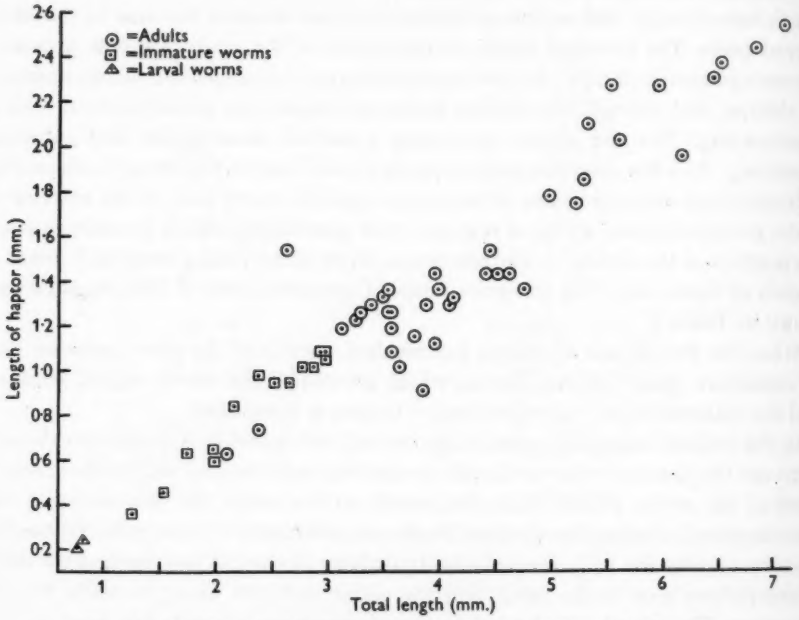

Fig. 11. Bodily proportions during growth: length/haptorial length.

haptorial length, transverse pharyngeal diameter and average transverse diameter of the three most anterior pairs of clamps have been used. Younger stages are not included because the length of the haptor was found to be more reliable for comparison with other organs than the total length and the haptor is, of course, not complete until the fifth larval stage. The coefficients of correlation are shown in Table 4.

Rate of development and longevity

The time relations of development during the first 7 hr. after the egg has been laid are shown in Fig. 4. The temperature at which eggs were kept during development was recorded and some were kept in a constant temperature bath. Since this bath was also in use for other purposes it could not be used for incubating eggs at abnormal temperatures. Such other temperatures, therefore, had to be chosen from those available and were liable to a certain amount of fluctuation. The effect of temperature on the rate of embryonic development is shown in Table 5.

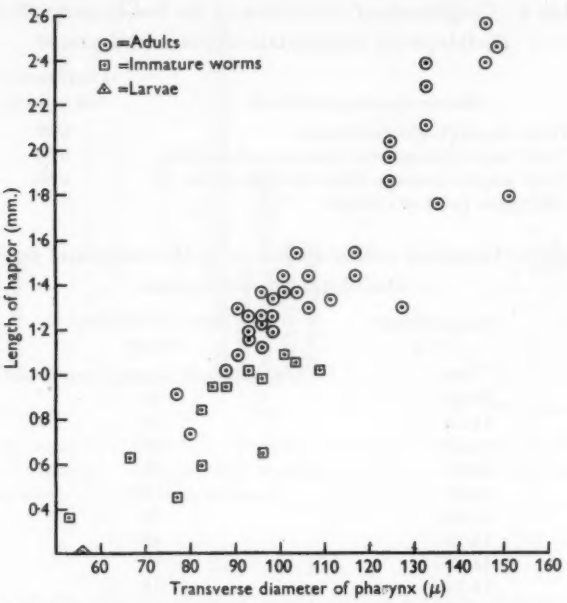

Fig. 12. Bodily proportions during growth: haptorial length/transverse diameter of pharynx.

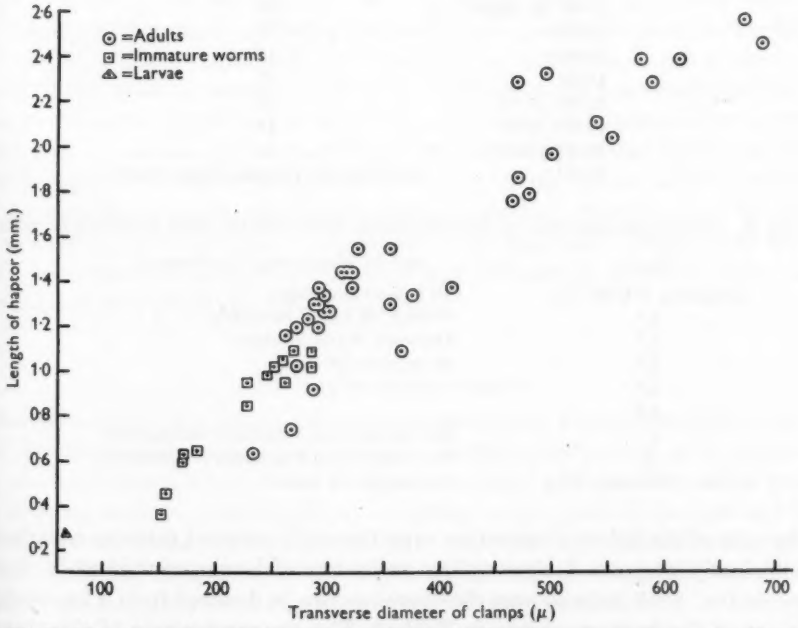

Fig. 13. Bodily proportions during growth: haptorial length/average transverse diameter anterior 3 pairs of clamps, or, in L^5, middle two pairs of clamps.

Table 4. *Coefficients of correlation of the bodily proportions of* Diclidophora denticulata *during development*

Measurements correlated	Coefficient of correlation
Total length/haptorial length	0·90
Total length/transverse diameter of pharynx	0·91
Total length/average transverse diameter of 1st three pairs of clamps	0·95

Table 5. *Variation with temperature of the embryonic period of* Diclidophora denticulata

Temperature (° C.)	Time to hatching (days)
7·00	Development began, then ceased
13·00	20
14·25	18
14·25	20
14·25	18
14·25	17
14·25	19
14·25	18
14·25	19
14·25	18
14·25	18
14·25–15·00	17
14·25–15·00	17
14·25 (7·00 1st night)	20
15·00	17
15·00	18
16·50	17
15·00–18·00	16
16·00–19·00	15
16·00–19·00	14
23·00	Development began, then ceased

Table 6. *Developmental rate of* Diclidophora denticulata *from hatching to maturity*

Stage	Age at which stage is attained
Hatching (14·25° C.)	18·3 days (average)
L^1	Within 24 hr. of hatching
L^2	Between 5 and 13 days
L^3	More than 38 days
L^4	—
L^5	—
I	Not more than 3 months (estimated)
A	Not more than 6 months (estimated)
A over 5·285 mm. long	21 months or more

In view of the failure to maintain experimentally infected fishes in aquaria over a sufficiently long period, observations on the rate of development must be, in part, speculative. Such facts as were discovered or can be deduced from a knowledge of the age of the host are shown in Table 6. The age composition of the observed population of flukes is shown in Figs. 14 and 15.

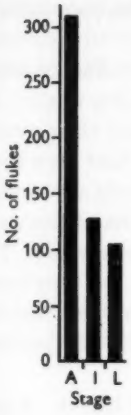

Fig. 14. Total stage-composition of population of *D. denticulata*.

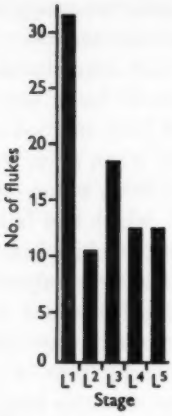

Fig. 15. Larval stage-composition of population of *D. denticulata*.

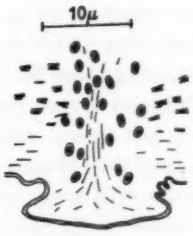

Fig. 16. L.S. body wall showing vaginal scar.

Reproduction

Despite much study of living worms, insemination was never seen. Accordingly, there is no direct evidence as to whether one or two individuals are involved or how it is effected. A few relevant facts are, however, noteworthy. Sperm was never observed in the uterus; on the other hand, in mature worms, sperm was found in the receptaculum seminis and also, frequently, in a scattered trail leading from there to the oviduct. Although the receptaculum seminis was never observed patent to the exterior, sections of mature worms revealed scar-tissue forming a break in the continuity of the layers of the ventral body wall, between the receptaculum and the surface of the body (Fig. 16). Other significant points are that infertile eggs are extremely rare, and that 12·8% of the mature worms collected and found to be producing eggs were solitary on the host: that is to say, no other worm, whether larval or mature, was found on the same host. Every adult worm which was examined showed ovary, testes and vitellaria in an active state, and reproduction took place throughout the year.

Effect of salinity variation

Though *G. virens* is a marine fish, it sometimes enters the mouths of streams to feed. For the present work, many fish were caught in the harbour at St Andrews into which the Kinnessburn flows. No general estimate of the salinity of the water can be given because it varies greatly according to the state of the tide and the amount of fresh water coming down the burn. In addition, the fresh water flows out on top of the salt water, so the degree of dilution to which the fish and its parasites are subjected must depend on their vertical position.

In view of the facts given above and since healthy specimens of *Discocotyle sagittata* were recovered from rainbow trout which had been acclimatized gradually

to sea water and thereafter kept for some months in sea water, the reactions of *D. denticulata* to dilution of the medium were tested.

Adult worms on removal from the host were placed in sea water diluted with an equal quantity of distilled water, controls being kept in normal sea water. After 24 hr., the two groups of worms were both in good condition and their eventual survival time was the same. However, when some worms which had been kept in 50 % sea water for 24 hr. were put into fresh water, cuticular blisters appeared and they died after about an hour. A fish, which had fallen into a tank of fresh water and remained there for a few minutes, was killed immediately afterwards. Three adult flukes were recovered from the gills in a paralysed condition, but they revived on being put in sea water. Moreover, a fish which was placed in fresh water for 10 min. and then returned to sea water, died 6 days later: an adult fluke producing eggs and a fifth-stage larva were recovered from its gills.

The effect of dilution of the medium was also tried on ciliated larvae. A group of larvae was divided into three lots: the first being kept in sea water as a control, the second being put into 50 % sea water and the third into distilled water, the minimum of sea water being transferred with the larvae to the experimental dish. The control larvae continued to swim vigorously throughout the experiment; those in 50 % sea water were moving slowly after 15 min. and were dead in an hour; those in distilled water swam actively for a short time, but soon fell to the bottom and were dead in 20 min. The flame cells of all were functioning normally till death occurred and there was no enlargement of the excretory ducts. In those subjected to dilution the cuticle rose in blisters before death.

Position and movement

Adult worms are always found lying along the inner face of a gill filament so that the worm's body rests between the two rows of filaments. Its position is such that, when at rest, the mouth is approximately level with the tip of the filament. The clamps, turned ventrally at right angles to the body, embrace the filament and each encloses a few gill lamellae in its grasp. Larger worms are, therefore, attached nearer the base of the filament on which they lie than smaller ones.

Larvae are much more variable in their choice of position. Their hooks and small clamps can grasp only lamellae on one side of a filament since the worm is too small to span the whole as adults do. Accordingly, they are usually found attached on one side of a filament between this and an adjacent one of the same row. Most commonly they are seen fairly near the tip of the filament. Larval worms, particularly early stages, move about much more than adults. First-stage larvae, when they are disturbed, loop over the gills from haptor to mouth in a leech-like manner, and all larvae strongly resist attempts to detach them from the host, clinging on with the mouth if the haptor is freed and taking a fresh grip with the haptor if possible. Adults, on the other hand, make little effort to resist detachment and never seem able to re-establish their hold if more than one or two clamps are dislodged. When they are watched on the excised gill the worms are usually fairly quiescent, the larvae making very characteristic, slight, jerking movements of the anterior region more or less regularly. Adults often gently shift the grip of

individual clamps without changing position. Sometimes they make exploratory movements with the neck region, apparently feeling their way over the surroundings with the mouth.

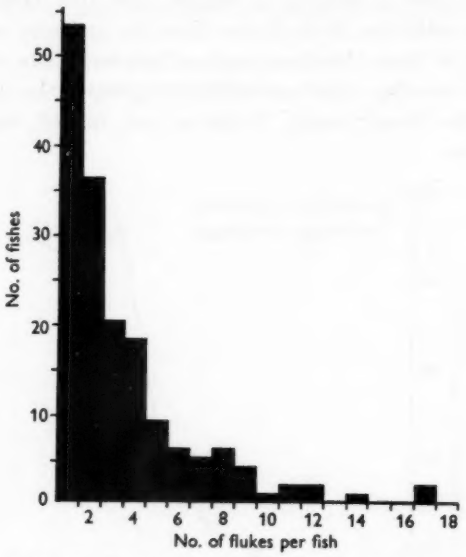

Fig. 17. Frequency of *D. denticulata* per host.

Table 7. *Distribution of* Diclidophora denticulata *per gill pair of the host*

Gill	No. of flukes	Percentage of total flukes	Adjusted percentage of total flukes
1st	223	40·77	39·59
2nd	195	35·65	34·62
3rd	64	11·70	11·36
4th	65	11·88	14·43

Degree of infestation and distribution on gills

In the course of the work, 269 host fishes were examined. Of these, 172, or 63·9%, were infested with *D. denticulata*. In estimating the average number of flukes per fish, 247 fishes were taken into account, the remaining 22 of the total number being discarded as no reliable count of the number of flukes they carried could be made. From among these 247 fishes, 554 flukes were recovered, giving an average of 2·24 flukes per fish, including in the total those fishes which were not parasitized. The frequency distribution among those which were parasitized, from 1–17 flukes per fish, is shown in Fig. 17. When considering the distribution of the flukes on individual gills, it must be taken into account that, though the first three pairs of gills are approximately equal in length, the fourth pair is only four-fifths the length of the others. Accordingly, in order to make the figures for the separate gills comparable, those for the fourth gill must be multiplied by 5/4. Table 7 shows the distribution per gill including adjusted percentages.

Pathogenicity

Sometimes gills with mutilated filaments occur both among infested and uninfested fishes, but there is nothing to suggest that this damage is in any way associated with the activities of the flukes since the majority of infested gills are healthy and perfect in form. Sections made of worms *in situ* on the gill filament reveal that even the lamellae which are within the grasp of the clamps show neither interference with the blood supply, irritation nor, indeed, any deviation from the normal condition.

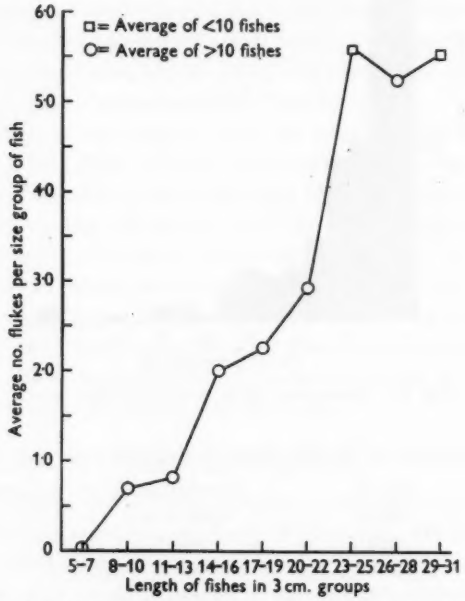

Fig. 18. Relationship between length of host and degree of parasitism by all stages of *D. denticulata*.

Host specificity and immunity

In order to distinguish between lack of opportunity for infestation and actual incompatibility with other hosts, an attempt was made to infest a young specimen of *Gadus callarias* with ciliated larvae of *D. denticulata* using the technique successfully employed with *Gadus virens*. The fish was killed on the following day and no larvae were found to have established themselves on the gills. There was, unfortunately, no opportunity of confirming this result by repetition of the experiment, but young specimens of *G. callarias* were often caught on the same feeding ground as *G. virens*. Many of these were examined, but *D. denticulata* was never found on their gills.

The relationship between degree of infestation and length (age) of host was examined as a measure of the possible development of age resistance, either natural or due to acquired immunity. Fig. 18 shows that there is an increase of infestation

with increase of length of host, the coefficient of correlation being 0·34. To distinguish between the capacity of the flukes to maintain themselves on the host as it gets older and the ability of larvae to establish themselves on older hosts, the relationship between the length (age) of host and the degree of infestation with

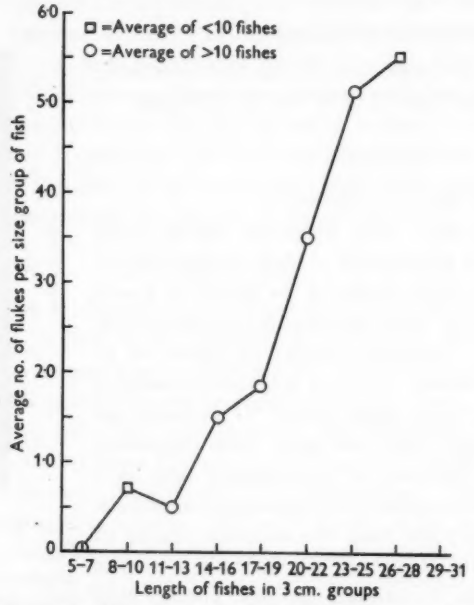

Fig. 19. Relationship between length of host and degree of parasitism by adult and immature *D. denticulata*.

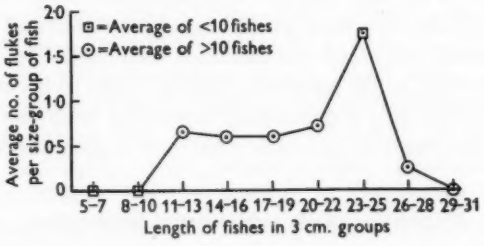

Fig. 20. Relationship between length of host and degree of parasitism by larval *D. denticulata*.

adult and immature worms separately was studied. Fig. 19 shows an upward trend of parasitism by adults with age, the coefficient of correlation being 0·37. Fig. 20 gives the corresponding situation in the case of immature and larval worms. Here the relationship is indefinite and the coefficient of correlation only 0·04.

To determine whether premunition occurs, a record was kept of the frequency of hosts carrying one or more separate infestations as indicated by the presence at the same time on the same host of different age groups of flukes. For this purpose,

it has been necessary to regard all adults as belonging to one age group, which patently they may not, owing to the impossibility of distinguishing between them by measurement since most of them were required for other aspects of the work. Fig. 21 shows that from one to seven separate infestations may be present at the same time.

Geographical distribution

D. denticulata is here reported, for the first time from St Andrews Bay and the Firth of Forth, Scotland.

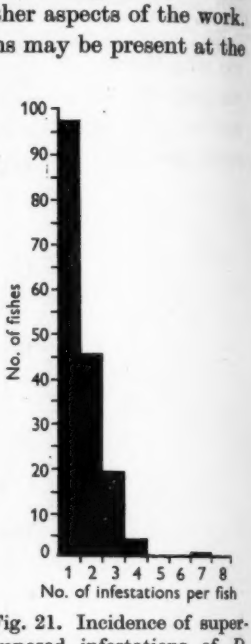

Fig. 21. Incidence of superimposed infestations of *D. denticulata*.

IV. DISCUSSION

The egg and oviposition

The form of the eggs here observed agrees with Cerfontaine's (1896) description of them in his study of *D. denticulata*. Two other species of the genus, *D. luscae* and *D. pollachii*, have been reported by Gallien (1934) to have eggs with filaments similar to those of *D. denticulata*, but evidently it is not a generic character since, according to Cerfontaine (1898), *D. palmata* has a non-filamented egg. There has been some discussion as to whether the filaments of monogenean eggs have specific functions or are merely accidental prolongations. Cerfontaine's (1896) view that the anterior crooks of the eggs of *D. denticulata* served to retain them on the gills is not borne out by present findings. Though eggs were sometimes found on the gills, they always proved to be new-laid ones and it must be concluded that these would have been expelled from the gill chamber if the host had not been killed just before they were laid. It is possible that the crooks may entangle the eggs in sea weed and may thus prevent them from being washed away from the haunts of their hosts. The only monogenean, other than *D. denticulata*, so far reported (Gallien, 1934) to lay its eggs in sheaves is *D. luscae*, but whether this is necessarily correlated with the presence of long posterior filaments bearing terminal disks could only be determined by further study of *D. pollachii*.

Maturation of the gametes and fertilization

Maturation of the spermatozoon could not be followed, the small size of the cells preventing adequate observations, but, in the oocyte, maturation is accompanied by reduction of the chromosome number from approximately eighteen to nine. Accordingly, nine is thought to be the haploid number of chromosomes in this species. Halkin (1901) reported that the chromosomes of *Polystoma integerrimum* were too small to examine in the second meiotic metaphase, but in *D. denticulata* the chromosomes of the two meiotic metaphases (Fig. 3) are about the same size. The second metaphase can be distinguished by the tendency of the chromatids to separate. That polar bodies have not been seen during the present work is not surprising in view of the reports in the literature of their ephemeral nature in other

trematodes. Whether entry of the spermatozoon stimulates maturation of the oocyte in *D. denticulata* cannot be said, but maturation of an oocyte was never observed prior to egg formation.

In *Polystoma integerrimum* Halkin (1901) and Goldschmidt (1902) found that the male and female pronuclei lay quiescent and separate until cleavage began, and the same has been reported for various Digenea (Schubman, 1905; Goldschmidt, 1905; Henneguy, 1906; Jones, 1933; Rees, 1939). On the other hand, Gille (1914) found that in *Gyrodactylus elegans* there was actual, irregular fusion of the karyomerites of the two pronuclei with the formation of a single, zygote nucleus. Unfortunately, the technical difficulties of cutting serial sections of the eggs, coupled with the extremely lobed form of the nucleus at this stage, make it impossible to say whether one or two nuclei are present in the zygote of *D. denticulata*.

Embryology

Fig. 4 shows that in *D. denticulata* cleavage does not always begin at the same interval after laying. Therefore, the onset of cleavage must be determined by the time of egg formation or sperm entry rather than by any stimulus, such as contact with sea water, which affects the whole sheaf of eggs at once.

The embryology of very few monogeneans has been followed owing to the technical difficulties arising from the impermeable nature of the egg-shell. Halkin (1901) has studied *Polystoma integerrimum*, and Katheriner (1904) investigated the special case of *Gyrodactylus elegans*. In both these, as in *D. denticulata*, total cleavage leads to the formation of an irregular morula in which germ layers cannot be distinguished. The early setting aside of the propagatory cell seen by Katheriner (1904) in *G. elegans* has not been observed in *Polystoma integerrimum* or, here, in *D. denticulata* and must be considered as a special adaptation to 'polyembryony in time'. That this lack of defined germ layers is common to other ectolecithal platyhelminths has been reported by Hyman (1951), who reviewed their embryology. Nuclei of rather differing appearance were seen in embryos of *D. denticulata*, but those of one type are not localized as Halkin found them to be in *Polystoma integerrimum*. It is thought that the differences seen in the present case depend on approaching mitosis rather than on any distinction into cell types. Perhaps the same explanation may apply to the indiscriminately scattered two types of cell noticed by Alvey (1936) in the late embryo of *Sphyranura oligorchis*. Halkin (1901) described the excavation of the larval gut sac in *Polystoma integerrimum* as taking place from the dorsal side, but in *D. denticulata* it almost certainly occurs from the ventral side. Yolk has several times been reported from the gut of young larval monogeneans. Paul (1938) saw it in the gut of larvae of *Polystoma nearcticum* as did Gallien & Le Calvez (1947) in *Kuhnia scombri*. Gallien (1935) stated that some yolk was swallowed by larvae of *Polystoma integerrimum* before they hatched, but Halkin (1901) made no reference to yolk in the gut. In view of the present findings in *D. denticulata* and Halkin's (1901) observations on gut formation in *Polystoma integerrimum*, it seems possible that Gallien was mistaken as to how the yolk entered the gut and that it may be incorporated as it is in *D. denticulata*. If, indeed, this is the case and proves on investigation of other species to be charac-

teristic of Monogenea, then it will be clear from Hyman's (1951) summary of the embryology of related platyhelminths, that monogenetic trematodes resemble rhabdocoel and alloeocoel turbellarians more closely in this respect than they do digenetic trematodes. The formation *in situ* of a ciliated epithelium here described for *D. denticulata* also occurs according to Halkin (1901) in *Polystoma integerrimum*. On the other hand, Halkin's findings in *P. integerrimum* with regard to a differential lining of the embryonic gut and the presence of temporary, anterior cavities were not found to have a parallel in *D. denticulata*, nor was a nerve band seen in the embryo of the latter species.

Fate of yolk

It has been observed that in new-laid monogenean eggs the yolk cells are numerous and clearly defined, but that as development proceeds they undergo degeneration and absorption by the embryo. This has been mentioned by Goldschmidt (1902) for *Polystoma integerrimum*, Siwak (1932) for *Ancyrocephalus vistulensis*, Wilde (1936) for *Neodactylogyrus macracanthus* and Paul (1938) for *Polystoma nearcticum*. A similar process occurs in *D. denticulata*, but it is not at all clear how it is brought about. Since disintegration of the yolk was seen to be delayed in eggs which did not contain embryos, it seems likely that some digestive ferment produced by the growing embryo may be the cause of the breakdown.

Hatching

Zeller (1872) noted that *Diplozoon paradoxum* larvae tended to hatch early in the morning, and Remley (1942) observed redoubled efforts to escape in larvae of *Microcotyle spinicirrus* when a powerful beam of light was turned on them, but there are no other reports of special hatching conditions. As to how the larva makes its escape there is little evidence of anything besides repeated pressure against the operculum (Kulwieć, 1927; Remley, 1942), though Paul (1938) suggested that cephalic glands might play their part in unsealing the operculum. From observations on *D. denticulata*, it is clear that considerable force is used by the larva which, owing to its length being greater than that of the egg, can press strongly on the operculum simply by attempting to straighten its body. This, repeated over and over again, may be sufficient to release the operculum though Paul's suggestion must not be ruled out.

The ciliated larva

Typically, the newly hatched monogenean larva is 'gyrodactyloid', a term proposed by Gallien (1934) implying an elliptical and dorso-ventrally flattened larva with bands of cilia, a suctorial mouth, bulbous pharynx, simple, sac-like gut, and a posterior, haptorial disk bearing various arrangements of hooks. This haptorial region is not, however, uniform throughout the group, but may be one of two types, or more or less intermediate between the two. The first type consists of a circular disk with a number of marginal hooks and perhaps one or two pairs of larger, median hooks. The second type of haptor consists of two lateral wings, each bearing hooks, and other hooks, often including a large pair, arising from the body of the larva medianly and posteriorly between the wings. The intermediate type has a disk, but there is a tendency for it to be drawn out laterally and for the hooks to be

arranged somewhat at the sides instead of being evenly distributed all round. This distinction between larvae might have either a phylogenetic or an adaptative significance. Accordingly, Table 8 has been compiled in which those gyrodactyloid larvae that have been described are arranged taxonomically, giving the haptorial type, and, as an indication of the conditions in which the haptor functions, the parasitic location of each. This table indicates that the division of the larvae into two groups by haptorial type accords more with the taxonomic position of the

Table 8. *Relationship between haptorial type in monogenean larvae, and their taxonomy and parasitic location*

Suborder and Superfamily	Species	Location	Haptorial type	Authority
Monopisthocotylea	*Neodactylogyrus macracanthus*	Gills	I	Wilde, 1936
Gyrodactyloidea	*Dactylogyrus anchoratus*	Gills	Intermediate	Kulwieć, 1927
	D. crassus	Gills	Intermediate	Kulwieć, 1927
	D. vastator	Gills	Intermediate	Kulwieć, 1929, Gröben, 1940
	Acolpenteron ureteroecetes	Ureters	I	Fischthal & Allison, 1941
	Ancyrocephalus vistulensis	Gills and skin	I	Siwak, 1932
Capsaloidea	*Benedenia melleni*	Skin and eyes	I	Jahn & Kuhn, 1932
Polyopisthocotylea	*Polystoma integerrimum*	Gill-chamber → bladder	I	Halkin, 1901
Polystomatoidea	*P. nearcticum*	Gill-chamber → bladder	I	Paul, 1938
	Polystomoides oris	Mouth	I	Paul, 1938
	Sphyranura oligorchis	Skin	I	Alvey, 1936
	Diplorchis scaphiopodis	(? →) Bladder	I	Rodgers, 1941
Diclidiphoroidea	*Kuhnia scombri*	Gills	II	Gallien & Le Calvez, 1947
	Microcotyle spinicirrus	Gills	Intermediate—II	Remley, 1942
	Diplasiocotyle johnstoni	Gills	II	Sanders, 1944
	Diclidophora luscae	Gills	II	Gallien, 1934
	Diclidophora pollachii	Gills	II	Gallien, 1934
	Diclidophora denticulata	Gills	II	Present work

species than with the site of parasitism. It would seem, therefore, that haptorial type is determined phylogenetically rather than as a larval adaptation. It is interesting to note that the division between the two types of haptor falls, not between the major groups Monopisthocotylea and Polyopisthocotylea, but that the whole of the Monopisthocotylea together with the Polystomatoidea of the Polyopisthocotylea form one group and the Diclidophoroidea of the Polyopisthocotylea comprise the other group. On this ground alone, then, there appears to be more affinity between the Monopisthocotylea and one superfamily of the Polyopisthocotylea than there is between the two superfamilies within the Polyopisthocotylea.

Apart from the present findings in *D. denticulata*, flame cells have only been described in two monogenean larvae: in *Microcotyle spinicirrus* by Remley (1942) and in *Benedenia melleni* by Jahn & Kuhn (1932). In addition to these species, however, a number have been described as having an excretory system, though flame cells were not referred to. Kulwieć (1927) mentioned this system in *Dactylogyrus crassus* and *D. anchoratus*. Halkin (1901) and Gallien (1935) both described an excretory system in *Polystoma integerrimum* as did Paul (1938) for *P. nearcticum*

and *Polystomoides oris* and Alvey (1936) for *Sphyranura oligorchis*. In at least some of the larvae where no excretory system has been described it is possible that further study might reveal it. Particularly may this apply to *Diclidophora luscae* in which Gallien (1934) found no excretory system. However, this larva is closely similar to that of *D. denticulata*, and in this latter species the excretory system was only eventually observed after much careful study of larvae compressed under coverslips.

The situation with regard to eye spots is different in that they are, if present, readily seen. They have been described in all the species of *Dactylogyrus* studied, in *Neodactylogyrus macracanthus*, *Ancyrocephalus vistulensis*, *Polystoma integerrimum*, *Diplasiocotyle johnstoni* and *Microcotyle spinicirrus* by the authors given in Table 8 and also in the larva of *Diplozoon paradoxum* by Zeller (1872). Thus, those with eyes are scattered through the group without reference to phylogeny or any other criterion so far detected. Further study may reveal that their presence is related to behaviour adapted to their use.

In addition to the larvae already discussed there are a few which deviate from the gyrodactyloid type. Thus, on hatching, *Sphyranura oligorchis* has been described by Alvey (1933, 1936) as being without a ciliated epithelium, and Zeller (1872) said that the newly hatched larva of *Diplozoon paradoxum* is already provided with the most posterior pair of definitive clamps. *Udonella caligorum* was said by Price (1938) to emerge from the egg as a small but perfect adult. These modifications can be interpreted as a progressive telescoping of the larval stages.

The refringent spheres here seen embedded in the parenchyma as well as in the gut of larval *D. denticulata* may correspond to those seen by Zeller (1872) in the larva of *Diplozoon paradoxum*. No explanation can here be offered for them, but it may be suggested that they might be fatty elements of yolk which have wandered between the cells of the larval tissue prior to absorption.

The ciliated larvae which have been described, and referred to above, all swim in much the same way, spiral rotation being a characteristic feature, while the non-ciliated larva of *Sphyranura oligorchis* was said by Alvey (1933, 1936) to swim by flapping the haptor, though Dawes (1946) stated that there is no free-swimming stage in this species.

Apart from Kulwieć's (1927) brief reference to the reaction of larvae of *Dactylogyrus crassus* and *D. anchoratus* to tapping of the dish, nothing has been found in the literature about the reactions to stimuli or means of finding a host displayed by monogenean larvae. The larvae of *D. denticulata* have here been observed to be completely unresponsive either to jarring or to light and shade stimuli. Vigorous larvae apparently are not chemically attracted to the host gills since they ignore pieces of fresh gill put in their dish, and it is open to doubt whether the tiring larvae, which were observed to settle on excised gill, did so from choice. It is unfortunate that technical difficulties prevented infestation of living fish while under observation. Larvae swimming near a potential host would naturally be drawn into the mouth in the respiratory current. No doubt this is how they reach the gills in the first place, but they would as soon be swept out again unless they responded actively and immediately on coming into contact with the gills.

Larval development

Larval development in monogenetic trematodes, so far as it is known, is characterized by growth, elaboration of the adult haptor in place of the larval one and the differentiation of any other specifically adult characters including, of course, the reproductive organs. The transformation of the larval to the adult haptor naturally involves least modification in the Monopisthocotylea, amounting largely, as has been shown by the work of Kulwieć (1927), Gröben (1940), Wilde (1936), and Jahn & Kuhn (1932) to growth of the whole and differentiation of the great hooks.

In the Polyopisthocotylea, a more radical metamorphosis of the haptor takes place. In most of the known cases each pair of suckers or clamps of the adult haptor is formed at the level of a corresponding pair of lateral, larval hooks. Gallien (1935) demonstrated this in *Polystoma integerrimum*, and Paul (1938) found the cases of *P. nearcticum* and *Polystomoides oris* to be parallel. *Diplozoon paradoxum*, studied by Zeller (1872), and now *D. denticulata* are diclidophoroideans found to follow this rule, but *Microcotyle spinicirrus* forms a striking exception. In Microcotylidae the number of clamps is considerable, and new pairs are added for some time after maturity is reached. Remley (1942) found the clamps of *M. spinicirrus* to be initiated anterior to the larval haptor and it seems probable from the preliminary work of Sanders (1944) that in *Diplasiocotyle johnstoni*, another microcotylid, clamps may be added both before and behind the first-formed ones. Sproston (1946) included in her diagnosis of the superfamily Diclidophoroidea a statement that the adult haptor is formed anteriorly to the larval one, but from the evidence presented above this seems more likely to be the exception rather than the rule. Gallien (1934), by analogy with the case of *Polystoma integerrimum*, suggested that the adult clamps of *Diclidophora luscae*, whose larva he described, would be formed round the lateral larval hooks. However, in view of the present findings that the clamps of *D. denticulata* are formed externally to the larval hooks, it seems probable that this will also prove to be the case in *D. luscae*.

D. denticulata conforms to the pattern of most other monogeneans (Zeller, 1872; Gallien, 1935; Paul, 1938) in that the reproductive organs are not differentiated until the adult haptor is complete, though cells destined to become vitellaria can be distinguished at a very early stage. Sproston (1945) has said that in trematodes it is usual for the testes to develop before the ovary, and this is borne out by the work of Alvey (1936) on *Sphyranura oligorchis*, Remley (1942) on *Microcotyle spinicirrus*, and Zeller (1872) on *Diplozoon paradoxum*. However, this does not occur in *D. denticulata* where ovary and testes mature simultaneously. Table 3 shows that maturity in *D. denticulata* is not attained at any definite size, there being an overlap in the lengths of immature and adult worms. This seems to be a common feature among trematodes and has been emphasized by Willey (1941) in his work on the digenean, *Zygocotyle lunata*.

The peculiar feature of alternative neotenic development has so far only been discovered in the genus *Polystoma* and, as Paul (1938) has pointed out, it is most unlikely that such an adaptation will be found in monogeneans whose hosts do not undergo metamorphosis.

Allometric growth has been reported by Gallien (1935) in *P. integerrimum*, by Kulwieć (1927) in *Dactylogyrus anchoratus* and by Sproston (1945) in *Kuhnia scombri*. Dawes (1946) and Sproston (1945) both thought this might be a common feature of trematode growth. However, as can be seen from Figs. 11–13, in *D. denticulata* from the fifth larval stage onwards the growth of the organs for which it was possible to obtain reliable measurements proved isometric.

The nature of the food in monogeneans and its association, in the Polyopisthocotylea, with the gut pigment, has for long been the subject of controversy (Goto, 1895; Fuhrmann, 1928; Gallien, 1935; Remley, 1942; Sproston, 1945; Dawes, 1946). Recently, Llewellyn (1954) has investigated the matter and come to the conclusion that polyopisthocotyleans feed mainly on blood, and that the pigment in the 'pigment cells' of the gut wall is haematin. The present findings are in accordance with his views. Cells found in the gut lumen of some larvae are thought to be partially digested red blood corpuscles and correspond exactly to the material from *Discocotyle sagittata* interpreted in a similar manner by Llewellyn. The fact that gut pigment has never been seen in unfed or very young larvae during the present work tends to confirm Llewellyn's statement that it is derived from the host's haemoglobin.

Rate of development and longevity

From a study of Fig. 4 the time which elapses in *D. denticulata* between egg formation and laying can be deduced. It is clear that since the eggs are laid in a sheaf some will have been retained in the uterus longer than others. As has already been observed, the onset of development does not depend on laying: therefore the first-formed eggs in a sheaf will undergo cleavage sooner than the last-formed ones. The graph shows that the first cleavage division may occur as soon as 15 min. after laying and these are presumably the oldest eggs in the sheaf. A small proportion of uncleaved eggs are found 5 hr. after laying, but 7 hr. after laying all eggs have undergone cleavage; these last-cleaving eggs will be the youngest eggs in the sheaf. The interval between cleavage of the oldest and of the youngest eggs in a sheaf will be the longest time which an individual egg may remain in the uterus: approximately 5 hr. No estimation of this period has been found in the literature concerning other monogeneans so no comparison can be made.

Turning to the duration of the embryonic period in Monogenea, it is seen from Table 9 that there is a wide range among the known species. Some adaptative significance with reference to the habits of the host, or conditions in which the parasite finds itself is probably involved here in all cases and certainly in connexion with 'winter eggs'. The rate of development is evidently, in some cases at least, influenced by temperature. The effects of varying temperatures on the embryonic development are shown in Table 5. It appears that some other factor may also operate here since there is a variability over a range of 2 days even when eggs are kept at the same temperature. The system breaks down altogether at more extreme temperatures. Indeed, it would not be expected that a marine animal, living as it does in conditions of relative stability, would have evolved any marked adaptations for survival of the eggs over more than a limited temperature range.

Once the larva has hatched, the free-swimming period, as shown in Table 10, is usually short. An anomaly occurs, according to Hess (1928), in an unspecified *Dactylogyrus*. Less evidence is available concerning the rate of development after

Table 9. *Duration of the embryonic period in Monogenea*

Species	Embryonic period (days)	Authority
Dactylogyrus crassus	3	Kulwieć, 1927
D. vastator	3–5 (var. with temp.)	Kulwieć, 1929
	2½ (20° C.)	Gröben, 1940
	4 (10–20°)	Gröben, 1940
D. anchoratus	5½ (20°)	Gröben, 1940
Neodactylogyrus macracanthus	5 (in warm conditions)	Wilde, 1936
	10–13 (in warm conditions)	Wilde, 1936
	5 (20°)	Gröben, 1940
Ancyrocephalus vistulensis	3	Siwak, 1932
Acolpenteron ureteroecetes	6–9	Fischthal & Allison, 1941
Diplorchis scaphiopodis	Hatches soon after laying	Rodgers, 1941
Microcotyle spinicirrus	5–11	Remley, 1942
Kuhnia scombri	*c.* 12	Gallien & Le Calvez, 1947
Polystoma integerrimum	17 (or more)	Halkin, 1901
	10–12 (20°)	Gallien, 1935
P. nearcticum	12–13	Paul, 1938
Diplozoon paradoxum	15	Zeller, 1872
Diclidophora denticulata	18·3 (14·25°)	Present work
Diplasiocotyle johnstoni	19	Sanders, 1944
Polystomoides oris	*c.* 28 (variable)	Paul, 1938
Sphyranura oligorchis	32	Alvey, 1933, 1936

Table 10. *Duration of the free-swiming stage in Monogenea*

Species	Free-swimming period	Authority
Dactylogyrus crassus	Few hr.	Kulwieć, 1927
D. anchoratus	Few hr.	Kulwieć, 1927
D. vastator	6–8 hr.	Gröben, 1940
Dactylogyrus 'sp.'	4–6 days	Hess, 1928
Neodactylogyrus macracanthus	4–5 hr.	Wilde, 1936
Benedenia melleni	*c.* 6 hr.	Jahn & Kuhn, 1932
Polystoma integerrimum	48 hr.	Gallien, 1935
P. nearcticum	48 hr.	Paul, 1938
Polystomoides oris	48 hr.	Paul, 1938
Sphyranura oligorchis	Few hr.	Alvey, 1933, 1936
Microcotyle spinicirrus	6–10 hr.	Remley, 1942
Diplasiocotyle johnstoni	48 hr.	Sanders, 1944
Diclidophora luscae	12–24 hr.	Gallien, 1934
D. denticulata	*c.* 24 hr.	Present work
Diplozoon paradoxum	6 hr.	Zeller, 1872

the larva has secured a host than in the earlier stages, but there are great differences in the few known cases, ranging from 3 days between attachment and maturity, as found by Gröben (1940) in *D. vastator*, to over 2 years, recorded by Gallien (1935) for the ordinary, slow development of *Polystoma integerrimum*, and varying with temperature. Between these extremes comes *Neodactylogyrus macracanthus*

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which Wilde (1936) found matured in a fortnight at 20° C. but, at 13–14° C., only reached maturity after 4½ weeks. Alvey (1933, 1936) noticed a temperature effect in *Sphyranura oligorchis*, but found this species usually matured after 2 months. Paul (1938) gave a minimum of a year before *Polystomoides oris* matured.

Only an estimate is possible in the case of *D. denticulata* owing to the failure to keep experimentally infested hosts throughout the developmental period of the flukes. Table 6 includes estimates of the times taken to reach the 'immature' and 'adult' stages. These are based on information of the ages of the hosts from which certain flukes were recovered. Some slight check of these estimates is provided by Fig. 14. This shows that the numbers of larvae of immature worms to be found in the sample populations are of the same order, and thus confirms the view that the larval and immature periods are somewhat the same length, that is, about 3 months each. Though less reliable than Fig. 14, owing to the numbers being smaller, Fig. 15 gives the stage composition of the larval population. From direct knowledge of the lengths of the first and second larval stages (Table 6), it is known that the first stage does not occupy the relatively long period implied in the graph. The drop in numbers of larvae recovered after the first stage probably indicates the comparative vulnerability of first-stage larvae. Once the larva has overcome the difficulties of accommodating itself to the host, there seems little cause for differential wastage in the subsequent larval stages. Accordingly, it can be taken that the durations of the second to fifth larval stages are proportional to the incidence of larvae of these stages, that is to say, approximately equal to one another, with perhaps a rather longer period spent in the third larval stage.

Very little appears to be known about the length of life of monogeneans. Gröben (1940) estimated the adult life of *Dactylogyrus vastator* at 1 or 2 days; and on the other hand, Gallien (1935) has found that *Polystoma integerrimum* can live for 6 or 7 years. No use can be made of Fig. 14 in this connexion, because the full picture cannot be seen when only the younger age groups of hosts have been examined.

Reproduction

Very little is known about insemination in Monogenea. Mutual cross-insemination has only been described (Zeller, 1872) in one instance, the special case of the permanently united *Diplozoon paradoxum*. In *Microcotyle stenotomi* mutual insemination is impossible because the cirrus is dorsal and the vagina ventral, but cross-insemination has been observed by MacCallum (1913). Wilde (1936) observed cross-insemination in *Neodactylogyrus macracanthus*, and Paul (1938) inferred that it was essential in *Polystomoides oris*. On the other hand, Gallien (1935) said that though cross-insemination is usual in *Polystoma integerrimum*, self-insemination can occur in isolated worms. Evidence on this matter gained during the present work is purely circumstantial as worms were never seen *in copula* or performing self-insemination. From the fact that infertile eggs are extremely rare, it is clear that, whatever the method, it is highly efficient. This high rate of fertility extended to the 12·8 % of the worms producing eggs which were solitary on their host. It is, of course, possible that this figure of 12·8 % is a distortion of the true facts as the

partner responsible for insemination might have lost its hold during the act and have been expelled from the gill chamber, or might simply have died after inseminating the remaining worm and before the host was caught and examined. However, since the 12·8% includes only those worms which were absolutely solitary on the host and takes no account of those which shared a host with larvae and immature worms incapable of providing spermatozoa, the figure is probably low rather than high. Accordingly, it must be concluded that, though self-insemination may not be the rule, it is highly probable that it can occur. Unfortunately it was not possible to prove this by rearing a worm to maturity in isolation from its congeners.

As to the method of insemination, again only speculations can be made. The fact that sperm has never been found in the uterus of *D. denticulata* does not exclude the possibility that it might pass through rapidly and so escape observation, but the presence of sperm in the seminal receptacle of adults as so constant a feature is highly significant. There is no communication between the male and female reproductive systems and the cirrus is well developed and armed in such a way that it could penetrate the body wall in making contact with the seminal receptacle. These factors, together with the presence of a scar between the seminal receptacle and the ventral body wall, indicate that sperm is deposited in the seminal receptacle by the cirrus piercing the body wall. The extensibility of the anterior region of the body would allow of the cirrus being apposed to the seminal receptacle of the same individual in the case of self-insemination. Odhner (1913) observed a similar 'temporary vagina' in *D. merlangi*.

Effect of salinity variation

Nothing has been reported of the function of the excretory system in Monogenea, but Hyman (1951) has suggested that this system is purely osmo-regulatory in Turbellaria and largely so in Digenea. Whether the excretory system of *D. denticulata* is concerned with excretion is not clear, but from the experiments on dilution of the medium mentioned earlier, it is evident that, by some means, a certain degree of osmo-regulation is possible in adults and will be important when the hosts feed at the mouths of rivers. However, this capacity does not extend to the ciliated larvae. This may be a factor in the greater mortality of first-stage larvae than of later stages which has been deduced from experimental evidence and Fig. 15.

Position and movement

The characteristic position of *D. denticulata* between the two rows of gill filaments is apparently common to the genus (Cerfontaine, 1896). From the literature it is clear that some monogeneans move about on the host a good deal (Wilde, 1936; Dawes, 1946), but this does not seem to be the case in *D. denticulata*. Young worms, particularly larvae, are more ready to move, and associated with this is the fact that they are more resistant to detachment than the adults are. As the fluke grows it must change its position, to keep the mouth at a suitable level, and it is possible that young worms may space themselves out rather than remain where they happened originally to have secured a hold.

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Degree of infestation and distribution on gills

Fuhrmann (1928) said that, generally speaking, the number of monogenean parasites on each host was small and Cerfontaine (1896) found that there were usually two or three individuals of *D. denticulata* on each host. These statements are in accordance with the present findings of an average of 2·24 worms per host, but the importance of examining a large number of hosts to arrive at an average figure is shown by the fact that the frequency of *D. denticulata* on *Gadus virens* individuals examined during the present work varied from one to seventeen (Fig. 17).

Preference for particular gills shown by Monogenea has occasionally been mentioned in the literature, Gröben (1940) speaking of it in *Dactylogyrus vastator* and Cerfontaine (1896) saying that *D. denticulata* was most commonly found on the second and third pairs of gills. Later (1898) he noted similar preferences in other species of the same genus. However, examination of large numbers has here shown that the first and second pairs of gills harbour about three times as many individuals of *D. denticulata* as do the third and fourth pairs (Table 7). No explanation of this situation can be suggested.

Pathogenicity

As a whole, monogenetic trematodes do little, if any, harm to their hosts (Goto, 1895; Dawes, 1946), but sometimes the holdfasts cause irritation or stasis (Kulwieć, 1927; Hess, 1928, 1930; Wilde, 1936; Gröben, 1940; Dawes, 1946), or, less certainly, damage may result from the worms feeding habits (Dawes, 1946). Jahn & Kuhn (1932) did not refer to the cause of the highly pathogenic effects of *Benedenia melleni* in their work on this species. It may be considered surprising that *D. denticulata*, which is thought, at least at times, to feed on blood, appears to have no harmful effect on the host. Apparently a balance has been struck and probably the worm sometimes contents itself with mucus. The quiescent behaviour of the mature worms will minimize irritation of the gills. Nevertheless, it is remarkable that the grip on the delicate gill lamellae does no damage and indicates a mutually satisfactory evolution of the haptor.

Host specificity and immunity

The fact that so many of the Monogenea do not harm their hosts is an indication of the old and stable relations between them. It is under conditions of this sort that close host specificity would be expected and is, in this group, found (Fuhrmann, 1928; Baer, 1934). *D. denticulata* has been recorded almost exclusively from the gills of *Gadus virens* (Olsson, 1875; Linton, 1901; Stafford, 1904; Cooper, 1915; Nicoll, 1915; Little, 1929; Rees & Llewellyn, 1941; Rees, 1953; Shelswell, unpublished), but Nicoll (1915) gave *G. minutus* as another host, and Baylis & Jones (1933) described an isolated case of finding it on *G. merluccius*. It has, however, been suggested (Rees, 1953) that in this last instance the host was incorrectly identified.

Unfortunately neither species of possible subsidiary host was available for study during the present work, but even if these are valid hosts, the ability of this worm to parasitize species of the genus *Gadus* does not, apparently, extend to *G. callarias*. The one negative result on attempting to infest experimentally a young fish with larvae would, in itself, be inconclusive, but the fact that *G. callarias* caught feeding with *G. virens* were not infested bears out the experimental findings. It seems, therefore, that true physiological specificity is involved here, rather than restricted opportunity for infestation or ecological specificity. Remley's (1942) work with *Microcotyle spinicirrus* suggests the same conclusion since he failed to infest experimentally fishes other than the normal host. Nigrelli & Breder (1934) likewise found that certain species of fish never acquired an infestation with *Benedenia melleni* in spite of exposure.

Species which act as host to a parasite may later develop immunity as a result of premunition, that is, no superinfection occurs; as a result of immunity acquired from a previous infection; or as a result of increased natural resistance, age resistance. The last-mentioned may, as Taliaferro (1940) has pointed out, be compounded of the second and third possibilities. Premunition has not so far been reported from Monogenea, and it is clear from Fig. 21 that it does not occur in the case of *D. denticulata*, where as many as seven separate infestations on one host have been observed.

Immunity as a result of previous infection has been recorded in some of the hosts of *Benedenia melleni* by Jahn & Kuhn (1932), Nigrelli & Breder (1934), and Nigrelli (1937), and also for some *Dactylogyrus* infections by Hess (1930). It is significant that these flukes are pathogenic.

Age resistance, however acquired, will lead to fewer parasites being found on larger, and therefore older, fishes. So far as *D. denticulata* is concerned, Fig. 18 shows that there is an increase in number of the flukes per fish with increasing host size and there is a significant, positive coefficient of correlation between the two values. It is recognized that all the fishes examined here were under 3 years old and that resistance might develop later. However, Llewellyn (unpublished) examined individuals of *Gadus virens* which were almost certainly more than 4 years old and yet harboured *D. denticulata*. It is, therefore, concluded that total immunity of *Gadus virens* to *D. denticulata*, does not occur either as a result of natural resistance increasing with age or of previous infestation with this species of fluke.

There is, however, a modification of the same principle to be taken into account. Quite apart from premunition, though parasites which are already present may be able to maintain themselves, larvae may fail to gain a hold. Fig. 20 shows that specimens of *Gadus virens* 27 cm. and over in length have not been found carrying larval *D. denticulata*. It is unfortunate that more fishes in the larger size groups were not obtainable. However, in spite of the inadequate numbers, the trend does seem downward.

There are three possible causes for failure of larvae to establish themselves on older fish. The first is ecological: the changed habits of the host fish, which involve its migration to deeper water, may mean that even the offspring of parasites it already carries have but slight chance of reaching a host. Secondly, there may be

increased natural resistance, and here may be included the mechanical fact that in larger fishes water will pass over more widely spaced gills, so that larvae will have less chance of attaching themselves as they are swept by. Thirdly, earlier infestation may have produced sufficient immunity to resist larval attack. In the present case, the first two of these possibilities are almost certain to operate, but the third seems less likely to occur since, in the absence of pathogenic effects, humoral response will probably not be provoked.

Geographical distribution

It is reasonable to assume that the distribution of monogenean parasites will correspond to that of their hosts (Fuhrmann, 1928; Dawes, 1946), and this is borne out by the records for *D. denticulata* and *Gadus virens*.

D. denticulata was first recorded on *Gadus virens* from the Skaggerak (Olsson, 1875), and Braun (1893) also gave this location, but was probably referring to Olsson's specimens. Records from the American side of the Atlantic include those of Linton (1901), Stafford (1904), and Cooper (1915). From the coasts of Britain records are given by Nicoll (1915), Little (1929), Baylis & Jones (1933), Rees & Llewellyn (1941), and Shelswell (unpublished), and in the present investigation. Rees (1953) has reported the species from Iceland, and Nicoll (1915) described it as occurring 'outside British waters'. On only two occasions has *Gadus virens* been examined parasitologically without finding *D. denticulata*. Manter (1926) examined only four fishes at Mount Desert Island, Maine; the presence of the fluke might well be found if larger numbers of hosts were examined. At Plymouth (Marine Biological Association Report, 1931) negative findings were also recorded but here again only a small number of fishes may have been examined since *Gadus virens* is recorded as 'occasional' in the area.

V. SUMMARY

1. Cerfontaine's (1896) description of the egg is confirmed. The eggs are not retained on the gills during development.

2. Maturation of the oocyte takes place after egg formation. The haploid number of chromosomes is 9.

3. The onset of embryonic development is related to the time of egg formation or sperm entry. Cleavage is total and slightly unequal, leading to the formation of a morula in which there are no germ layers. The larval gut is formed by epiboly round a mass of yolk which becomes incorporated in mid-body. Larval organs and a partially ciliated epithelium are differentiated *in situ*.

4. During embryonic development yolk is broken down and absorbed, some being incorporated in the body.

5. Hatching does not seem to result from any special stimulus. Mechanical pressure by the larva forces off the operculum, but secretions may weaken the suture.

6. The free-swimming larva is gyrodactyloid with a winged haptor bearing four pairs of lateral, one pair of postero-lateral and one pair of median hooks. Four pairs of flame cells are present and one pair of longitudinal, lateral, excretory

ducts, which open antero-laterally. The larva swims by ciliary action and does not respond to jarring, light and shade stimuli, or, when vigorous, to the presence of the excised gill of the host. Tired larvae are doubtfully attracted by host gill tissue. Larval haptorial type is thought to be determined phylogenetically rather than as a result of adaptation to the type of host tissue to which the larva attaches itself.

7. The larva attaches itself to the host's gill and sloughs off the ciliated coat. Growth follows with the development of the adult clamps in pairs, starting posteriorly, each pair replacing a pair of lateral, larval hooks. Primordia of the reproductive organs are laid down in the larval stage, but are only finally elaborated when the adult form has been attained. Maturity is not reached at a definite size and the ovary and testes mature simultaneously.

8. Feeding is at least partly on the host's blood.

9. The longest time which may elapse between formation and laying of an egg is about 5 hr. The average time taken for embryonic development is 18·3 days at 14·25° C., but temperature affects the developmental rate. Ciliated larvae survive about 24 hr. in the absence of a host. The second larval stage is reached between 5 and 13 days after hatching, the third larval stage after more than 38 days. The immature stage is thought to be reached within 3 months and the adult stage within 6 months.

10. Reproduction occurs throughout the year. The animal is functionally hermaphrodite at all times once maturity is reached.

11. A certain degree of osmo-regulation is possible in adults, but this does not extend to ciliated larvae.

12. Adults rest along a gill filament on its inner face and do not move about. Larvae are more ready to move.

13. The average number of flukes per host is 2·24. The 1st and 2nd pairs of gills carry three times as many flukes as the 3rd and 4th pairs.

14. The host suffers no apparent harm from the presence of the flukes.

15. Premunition of *Gadus virens* against *Diclidophora denticulata* does not occur. Age resistance is probably developed to the extent that larvae cannot establish themselves on older fish and is probably due to mechanical difficulties and changed habits of the host. The ability of this fluke to parasitize species of *Gadus* does not extend to *G. callarias*.

16. *D. denticulata* is recorded for the first time from St Andrews Bay and the Firth of Forth.

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A REVIEW OF THE LAELAPTID PARAPHAGES OF THE MYRIAPODA WITH DESCRIPTIONS OF THREE NEW SPECIES (ACARINA: LAELAPTIDAE)

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(With 30 Figures in the Text)

The Laelaptidae undoubtedly show more variety in their external morphology than any other family of the Mesostigmata. This is due largely to the specialized structures of those species which have adopted a parasitic mode of life, either as ecto- or endo-parasites of mammals, birds and reptiles, or as paraphages of arthropods. The structural modifications apparent in the parasitic forms tend to conceal the true relationships between these forms and non-specialized free-living members of the family which, although more numerous, have received considerably less attention from the systematist. The object of the present work is to review our knowledge of a small group of Laelaptid mites associated with Chilopoda and Diplopoda and to discuss their relationships with other members of the family.

SYSTEMATICS

Vitzthum (1941) refers to eight species of Laelaptid mites, from four genera, as having been recorded from Myriapoda. These genera are distributed in two sub-families, the Hypoaspidinae and the Iphiopsinae; the former containing *Hypoaspis* Can. and *Julolaelaps* Berl., and the latter *Iphiopsis* Berl. and *Jacobsonia* Berl. These four genera and a new genus described below may be separated as follows:

1. Peritreme normal, extending beyond coxa I. 2
— Peritreme reduced or absent, at the most reaching the middle of coxa III. 3

2. All legs terminating in a pulvillus and two claws. **Hypoaspis** Can. s.lat.
— All legs terminating in a pulvillus without claws. **Julolaelaps** Berl.

3. Claws on leg I absent; claws on legs II–IV normal. **Iphiopsis** Berl.
— Claws on all legs absent. 4

4. Dorsal shield not completely covering the dorsum of the mite; sternal shield strongly sclerotized; gnathosoma without external posterior rostral setae; anal shield with two setae. **Jacobsonia** Berl.

— Dorsal shield completely covering the dorsum of the mite; without sclerotized sternal shield; gnathosoma without capitular setae; three setae on the anal shield. **Scolopendracarus** n.gen.

Genus **Iphiopsis** Berl. 1882

1882. *Iphiopsis* Berlese, A., *A.M.S.* Fasc. 1, No. 4, figs.

The genus *Iphiopsis* was erected by Berlese with *Iphis miriabilis* Berl. 1882 as the type species. This is the only species of the genus and has not been found

subsequently. The definition of the genus must be based, therefore, on the short description of the type. The most important characters of *I. miriabilis* are the reduction of the peritreme—a character used by Berlese (1892) to separate *Iphiopsis* from other 'genera Laelaptidarum'—and the absence of claws on leg 1. The sternal and genital shields are well developed, and the anal shield bears three setae. The digits of the chelicerae are dentate and of equal size. Only the female is known.

The habitat is stated to be 'in humus and on *Iulus varius* Fab., Patavii, rare'.

Genus **Jacobsonia** Berl. 1910

1910*a*. *Greeniella* Berlese, A., *Redia*, **6**, 247 (nom. praeocc.).
1910*b*. *Jacobsonia* Berlese, A., *Redia*, **6**, 373 (nom. nov. pro. *Greeniella* Berl. 1910).

The genus *Jacobsonia* s.lat. may be defined as follows:

Dorsal shield in both sexes not completely covering the dorsum of the mite. In the female the sternal shield is strongly sclerotized and bears three pairs of setae. The genital shield may be large and extending as far as the anal shield or reduced and reaching only a short distance beyond the posterior margin of coxae IV. The genital orifice, a transverse slit in the region of coxae IV, is covered by a hyaline extension of the genital shield. In the male the fused sterniti-genital shield extends from the posterior margin of coxae I to the middle of coxae IV, and the genital orifice is situated on its anterior margin. The anal shield in both sexes is pear-shaped and carries only the para-anal setae. The stigmata are situated ventro-laterally in the region of the third inter-coxal space. The peritreme is strongly reduced or absent in both sexes. The external posterior rostral setae are absent and the palpgenu bears five setae only. The chelicerae are dentate in both sexes and of approximately equal size. The legs are stout and all terminate in a pulvillus without claws. Tarsus II, in the known males, is spinate.

Type: **Iphiopsis (Greeniella) submollis** Berl. 1910

This genus may be divided into two subgenera on the form of the genital shield and the peritreme in the female.

1. Genital shield markedly reduced, barely extending beyond the posterior margin of coxae IV. Peritreme absent. (Malaya and Java.) **Jacobsonia** s.str.
— Genital shield well-developed, extending almost to the anal shield. Peritreme present but reduced and extending only to the posterior margin of coxae III. (West Africa.) **Parajacobsonia** n.subgen.

Subgenus JACOBSONIA Berl. s.str.

This subgenus contains three species; two described by Berlese from Java and a new species described below from Malaya.

1. Region posterior to the dorsal shield in the female without setae. **J. (Jacobsonia) submollis** Berl.
— Region posterior to the dorsal shield with four pairs of setae. 2
2. Movable digit of the chelicera in the female with an anteriorly directed process; spermatophoral process in the male elongate, lanceolate; without (?) marked sexual dimorphism in the chaetotaxy of the dorsal shield. **J. (Jacobsonia) minor** Berl.

— Movable digit of the chelicera in the female without a process; spermatophoral process in the male clubbed; strong sexual dimorphism in the chaetotaxy of the dorsal shield.
J. (Jacobsonia) audyi n.sp.

Jacobsonia (Jacobsonia) submollis (Berl.) 1910

1910*a*. *Iphiopsis* (*Greeniella*) *submollis*, Berlese, A., *Redia*, **6**, 248.
1910*b*. *Jacobsonia submollis*, Berlese, A., *Redia*, **6**, 373.
1913. *Jacobsonia submollis*, Berlese, A., *Redia*, **9**, 80 figs.

The original description of *J.* (*J.*) *submollis* is as follows:

Foem. testacea, cordiformis, postice acuta, glaberrima; scuto dorsuale vix plus quam dimidiam antiem partem abdominis obtegente. Pedes et rostrum ut in gen. *Celaenopsis*. Ad 800μ long.; 500μ lat.
Habitat supra *Scolopendra* sp., 'Giava'.

From the characters given in the definition of the subgenus by Berlese the following may be added to the above description: 'Peritremata nulla.... Mandibulae stylo longo ad summum digitum fixum...Scutum genitale minimum....'

In 1913 Berlese figured the dorsum of the type female. The chief character brought out in the figure is the absence of setae postero-dorsally. The writer has used this character in the above key to separate *submollis* from *minor* and *audyi*.

Jacobsonia (*Jacobsonia*) *minor* Berl. 1910

1910*b*. *Jacobsonia minor*, Berlese, A., *Redia*, **6**, 374.
1913. *Jacobsonia minor*, Berlese, A., *Redia*, **9**, 80 figs.

The original description of this species is as follows:

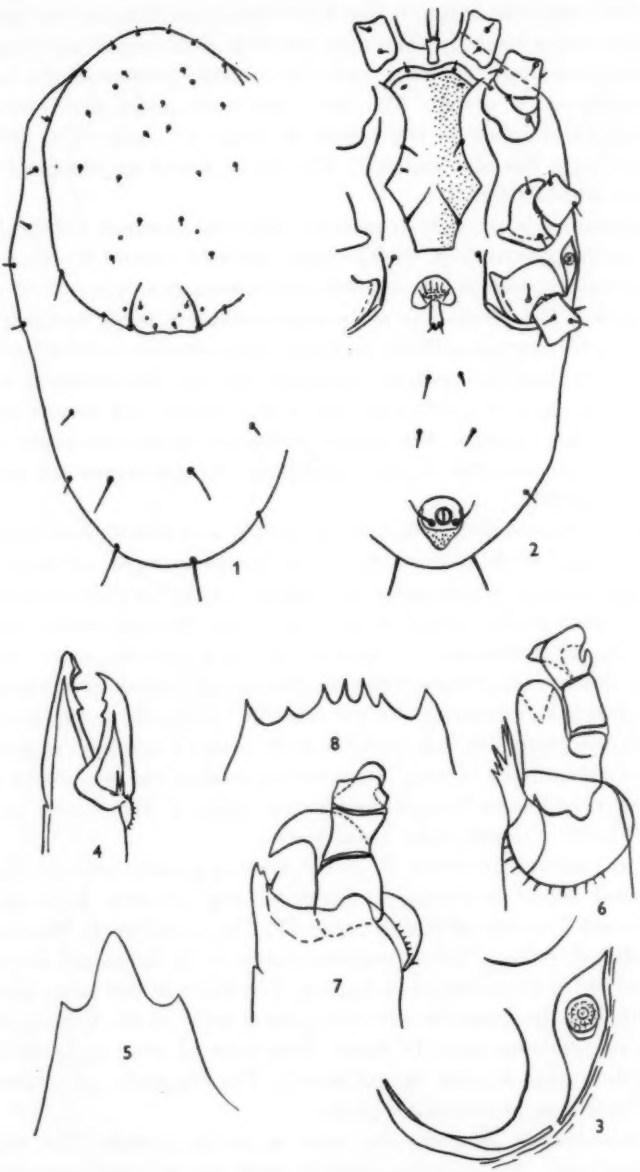
J. submolli omino similis, sed multo minor. Mas calcare mandibulae bistiligero, stylo recto antrorsus directo, peracuto, alio basali recurrenti, retrosus directo. Pedes secundi paris crassi, tarso conico, tuberculis duobus in latere alioque apicali. Foem. ad 670μ long.; 400μ lat. (ovigera); Mas ad 550μ long.; 350μ lat.
Habitat 'Giava, Wosonobo', super Scolopendram sp. cum *Heterozercon audax*. Collegit Clar. Jacobson.

Both sexes of this species were figured by Berlese (1913).

Jacobsonia (*Jacobsonia*) *audyi* n.sp.

Female. Dorsal shield, oval in contour measuring 430μ in length and 308μ in breadth, bears sixteen pairs of simple setae arranged as in Fig. 1. It covers the anterior part of the body only. The setae are very short, with the exception of two pairs situated on the posterior margin. The interscutal membrane lateral to the shield carries five setae. The remainder of the dorsal chaetotaxy comprises four pairs of simple setae situated on the striated cuticle posterior to the dorsal shield. The relative position of these setae varies considerably in the material examined.

Ventrally the tritosternum is well developed and has long smooth lacinae (Fig. 2). The sternal shield, strongly sclerotized and minutely punctured, extends from the posterior margin of coxae I to the posterior margin of coxae III. The three pairs of sternal setae of which the third pair is the longest, and two pairs of 'pores' are distributed as in the figure. The short metasternal setae lie on the interscutal membrane postero-lateral to the sternal shield. The genital orifice is situated between coxae IV and is covered by a hyaline expansion of the much reduced



Figs. 1–8. *Jacobsonia* (*Jacobsonia*) *audyi* n.sp.

Fig. 1. Dorsal of female. Fig. 2. Ventral of female. Fig. 3. Stigma and peritreme of female. Fig. 4. Chelicera of female. Fig. 5. Tectum of female. Fig. 6. Chelicera of male. Fig. 7. Chelicera of male (ventral). Fig. 8. Tectum of male.

genital shield. The latter bears two pairs of short, simple setae. The region between the genital and anal shields is provided with three pairs of setae, two pairs medially and one pair ventro-laterally. The anal shield is pear-shaped and bears one pair of setae—the para-anals. The post-anal seta normally present on the anal shield in the Laelaptoidea is absent. The exo- and endo-podal plates are markedly reduced, being developed in the region of coxae IV only. The peritreme and peritrematal plate are also reduced. The latter forms an elongated plate surrounding the stigma (Fig. 3).

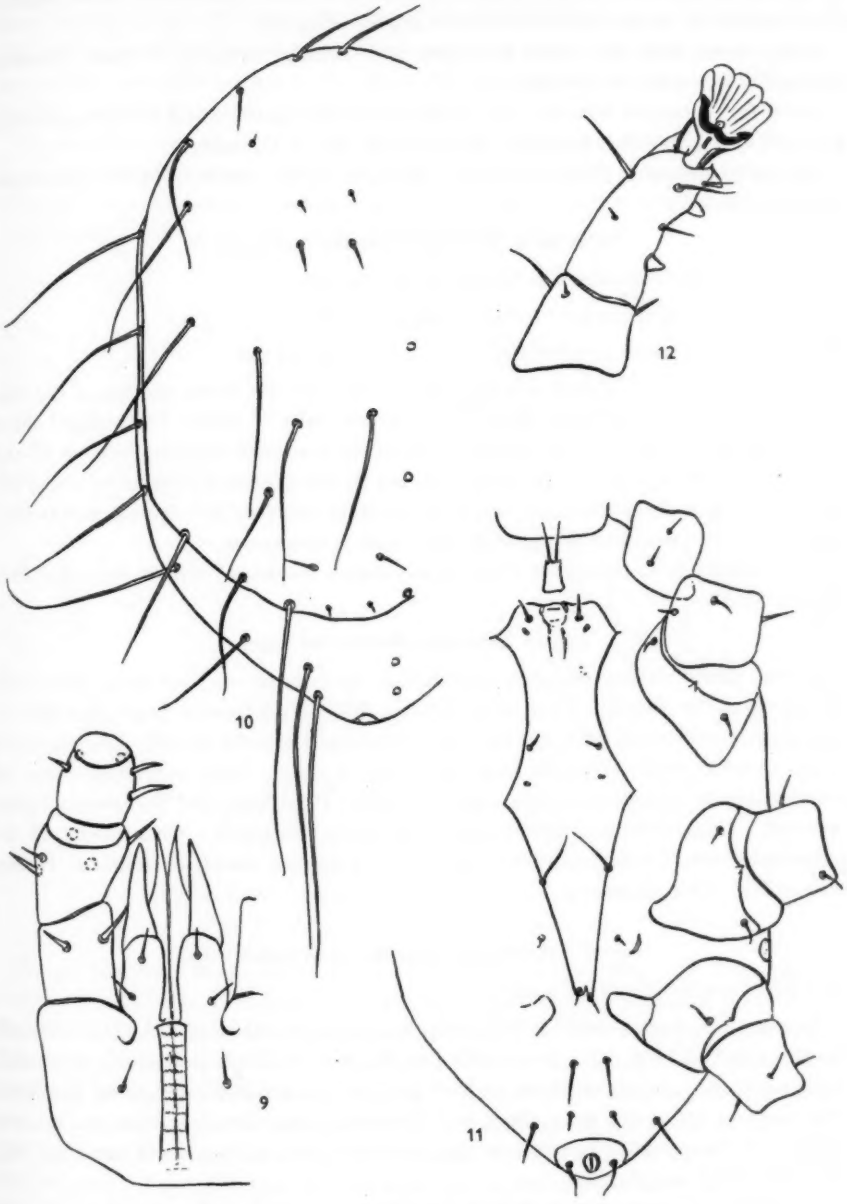
The gnathosoma is strongly sclerotized and the corniculi extend beyond the middle of the palpfemur (Fig. 9). The three pairs of ventral setae are short. The posterior rostral setae are not developed. The ventral groove is provided with eight rows of denticles. The chaetotaxy of the palptrochanter, femur and genu is (2–5–5). The chelicerae are dentate with the digits of approximately equal length. The fixed digit is tridentate and the movable bidentate (Fig. 4). The former is not provided with a process as in *J.(J.) submollis* and *J.(J.) minor*. The tectum (epistome) is produced into three prongs; the lateral prongs are short and acute, the median prong long and obtuse (Fig. 5). In a number of the paratypes the median prong is denticulate distally.

The legs are well-sclerotized and all terminate in a pulvillus without claws.

Male. The dorsal shield ($440 \times 320\mu$) covers the greater part of the dorsum in the male. The dorsal setae are considerably longer than in the female with the exception of three pairs on the dorsal shield. The shield bears seventeen pairs of setae (Fig. 10). The additional pair of setae in the male (sixteen in the female) is the result of the fusion of the bases of the anterior pair of lateral interscutal setae with the dorsal shield. The remainder of the setae on the lateral interscutal membrane in the female are present in that position in the male. Posterior to the dorsal shield there are only three pairs of setae; the external setae of the second row of posterior dorsal setae in the female being absent in the male (cf. Figs. 1 and 10). There are twenty-four pairs of dorsal setae in the male.

The region posterior to coxae IV in the male is greatly reduced (Fig. 11). The sterniti-genital shield is strongly sclerotized and extends from the posterior margin of coxae I to the middle of coxae IV. The shield bears four pairs of setae and two pairs of 'pores'. The metasternal setae lie off the shield and are situated in the region of the third inter-coxal space. The third pair of setae are the longest and the fifth pair, homologous with the genital setae of the female, the shortest. The region posterior to coxae IV bears three pairs of setae as in the female. The anal shield is similar to that in the female. The stigmata are situated postero-laterally. There is no peritrematal plate.

The gnathosoma is basically the same as in the female. The chelicerae are strongly developed. The movable digit is hook-like and without teeth (Fig. 6). It bears a three-segmented spermatophoral process. The basal segment is fused with the digit; the terminal segment is complex in form and is shown in ventral view in Fig. 10. The movable digit is reduced and resembles that digit in *Blattisocius dentriticus* (Berl.). It is unidentate and bears a strong *pilus dentilis*. The tectum is denticulate (Fig. 8).



Figs. 9–12. *Jacobsonia* (*Jacobsonia*) *audyi* n.sp.
Fig. 9. Gnathosoma of female (ventral). Fig. 10. Dorsal of male. Fig. 11. Ventral of male. Fig. 12. Tarsus II of male.

All legs terminate in a large pulvillus without claws. Tarsus II, as in the male of *J.*(*J.*) *minor*, is armed with three stout spines (Fig. 12).

Dimensions. Male 480–500μ in length, 300–320μ in breadth. Female 740–800μ in length, 400–430μ in breadth.

Locality. Nineteen females and eight males on a giant Julid (*Thyropygus* sp.) Fraser's Hill (4000 ft.), Pahang, Malaya. Coll. Dr J. R. Audy.

Holotype female (1954:6:14:1), allotype male (1954:6:14:2), paratypes (1954:6:14:3–7).

Subgenus **Parajacobsonia** n.subgen.

This subgenus contains the following species only:

Jacobsonia (*Parajacobsonia*) *tertia* Vitzthum, 1931.

1931. *Jacobsonia tertia*, Vitzthum, H. G., *Zool. Anz.* **96**, 153 figs.

In this species the dorsal shield is larger than in the three species of the subgenus *Jacobsonia* and bears more than sixteen pairs of setae. The genital shield covers the greater part of the region between the posterior margin of coxae IV and the anal shield. The short peritreme reaches to the posterior margin of coxae III. The fixed digit of the chelicera bears an elongate process. The female is 800–885μ in length and 445–460μ in breadth. The male is unknown.

J.(*P.*) *tertia* is known only from West Africa (Gaboon) and is recorded from 'Myriapoda'.

Genus **Scolopendracarus** n.gen.

Dorsal shield entire, strongly sclerotized, reticulated and covering practically the whole of the dorsum of the mite. Dorsal shield with twenty-four pairs of setae. Tritosternum normal with lacinae. No sclerotized sternal shield. Genital shield large, convex posteriorly and bearing a pair of setae. Legs short and stout; all terminating in a large pulvillus without claws. Peritreme and peritrematal plate reduced. Gnathosoma without capitular setae ventrally. Chaetotaxy of the palptrochanter, femur and genu (2–4–4). Chelicerae chelate—dentate. Tectum triangular. Male unknown.

Type: **Scolopendracarus brevipilis** n.sp.

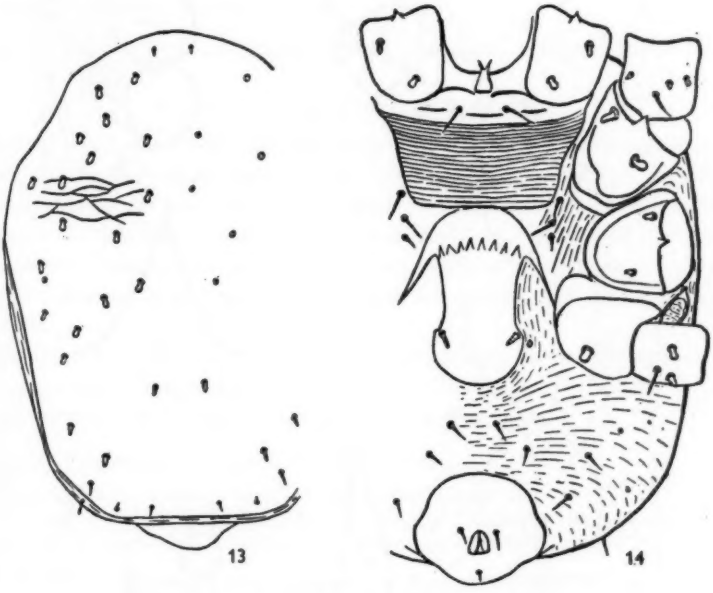
Scolopendracarus brevipilis n.sp.

Female. The dorsal shield is 347–450μ long and 297–340μ broad. It is widest in its anterior half (Fig. 13). Anteriorly the shield is rounded, posteriorly truncated. The chaetotaxy comprises twenty-four pairs of setae distributed as in the figure. The vertical setae are very short and situated some distance from the anterior margin of the shield. The setae in the posterior third of the shield are spine-like, the remainder are clubbed.

Ventrally, the sternal region is occupied by transversely striated cuticle (Fig. 14). The sternal setae are not situated on the transversely striated region. The writer was unable to see any 'pores'. The metasternal setae are considerably shorter than setae II and III. The tritosternum shows marked variation in form (cf. Fig. 15). The genital shield extends a short distance beyond the posterior margin of coxae IV.

It is slightly indentated laterally and convex posteriorly. The genital setae are short and spine-like. The anal shield is approximately as long as broad and bears the para-anals and a postanal seta. These setae are simple. The anal shield projects beyond the posterior margin of the body. The region between the genital and anal shields is provided with two pairs of setae.

The stigmata are situated ventro-laterally in the region of coxae IV. The peritreme is markedly reduced and reaches as far as the posterior margin of coxae III (Fig. 16). The peritrematal plate is poorly developed and is not attached to the dorsal shield. The exo-podal plates are also reduced (Fig. 16).



Figs. 13–14. *Scolopendracarus brevipilis* n.sp. Female.
Fig. 13. Dorsal view. Fig. 14. Ventral view.

The gnathosoma bears three pairs of setae ventrally (Fig. 17). The internal posterior rostrals are considerably longer than the external posterior rostrals and the rostral setae. The capitular setae are absent. The ventral groove is provided with six rows of denticles. The corniculi extend as far as the anterior margin of the palpfemur and are hooked distally (Fig. 18). The specialized setae on the palptarsus is two-pronged. The tectum is well-developed and triangular in shape (Fig. 19). The chelicerae show a certain degree of specialization. The fixed digit, slender and without teeth, is considerably shorter than the movable digit (Fig. 20). The latter is tridentate.

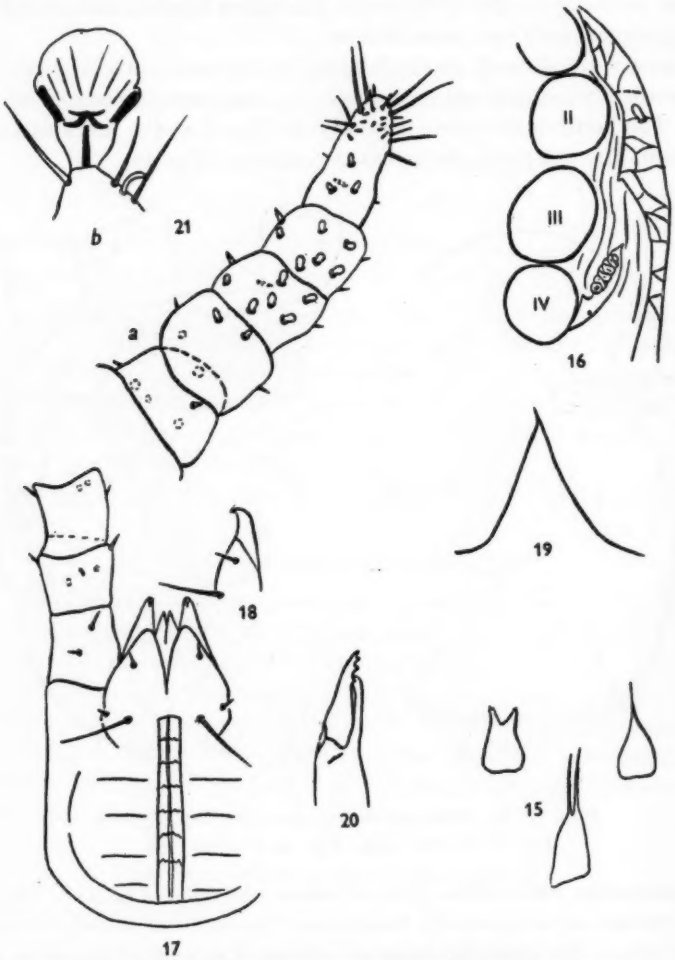
The legs are strongly developed and all terminate in a large pulvillus without claws (Fig. 21). The legs are richly provided with short club-like setae (Fig. 21).

The male is unknown.

Dimensions. Female: 365–470 μ in length, 297–340 μ in breadth.

Locality: Three females taken from *Scolopendra morsitans* (Linn.) on Aru Island (west of New Guinea). Collected in August 1902.

Holotype (1954:6:14:28), paratypes (1954:6:14:29–30).



Figs. 15–21. *Scolopendracarus brevipilis* n.sp. Female.

Fig. 15. Tritosternum, showing variation in structure. Fig. 16. Lateral view of peritreme and peritrematal plate. Fig. 17. Gnathosoma (ventral). Fig. 18. Lateral view of corniculus. Fig. 19. Tectum. Fig. 20. Chelicera. Fig. 21. *a*, Leg I (without ambulacral apparatus); *b*, ambulacral apparatus of Leg I.

Genus **Julolaelaps** Berl. 1916

1916. *Julolaelaps*, Berlese, A., *Redia*, **12**, 31.

This genus was erected by Berlese (*loc. cit.*) for a small group of mites living on Julids. In the definition of the genus he states that the species resemble very closely those of the genus *Hypoaspis* but lack claws on all legs. The male is known only for the genotype, *Julolaelaps dispar* Berl.

The following three species are known:

Julolaelaps dispar Berl. 1916

1916. *Julolaelaps dispar*, Berlese, A., *Redia*, **12**, 31.

Habitat. On Julid, Italian Somaliland (Goriei).

Julolaelaps rotundatus Berl. 1916

1916. *Julolaelaps rotundatus*, Berlese, A., *Redia*, **12**, 32.

Habitat. On Julid, Italian Somaliland (El Ualac).

Julolaelaps lucator Berl. 1916

1916. *Julolaelaps lucator*, Berlese, A., *Redia*, **12**, 32.

Habitat. On Julid with *J. dispar*, Italian Somaliland (Goriei and El Ualac).

Genus **Hypoaspis** Can. 1885 s.lat.

The genus *Hypoaspis* s.lat. has been divided into a number of subgenera of which a few are of uncertain systematic status. Species of two subgenera, *Hypoaspis* and *Coleolaelaps*, have been found on Myriapoda. They show little or no modification in external morphology compared with the free-living species of the genus. The chelicerae are chelate-dentate in both sexes, there is no reduction in size of the shields; the peritreme and peritrematal plate are normal and the legs are provided with claws.

Vitzthum (1941) records two species of *Hypoaspis* from Myriapoda. A third species, *Hypoaspis* (*Coleolaelaps*) *amazon* Berl. 1920, was overlooked by Vitzthum. A fourth species, new to science, is described below.

Hypoaspis (*Hypoaspis*) *spirostrepti* Oudms. 1914

1914. *Hypoaspis spirostrepti*, Oudemans, A. C., *Ent. Ber.* **4**, 69.
1915. *Hypoaspis spirostrepti* Oudemans, A. C., *Archiv. Naturgesch.* **81**, 131 figs.
1921. *Hypoaspis spirostrepti* Vitzthum, H. G., *Archiv. Naturgesch.* **86**, 14 figs.

The female is described and figured by Oudemans (1915) and the male by Vitzthum (1921). The former states that the female is ovoviviparous or viviparous.

H. spirostrepti was first collected from a living *Spirostreptus* sp. (from 'German East Africa') in Amsterdam. Later Vitzthum records it from a *Spirostreptus* sp. (from India) in Hamburg.

Hypoaspis (*Hypoaspis*) *indicus* Vitzthum 1921

1921. *Laelaps* (*Hypoaspis*) *indicus*, Vitzthum, H. G., *Archiv. Naturgesch.* **86**, 16 figs.

Both sexes of this species are described and figured by Vitzthum (*loc. cit.*).

Habitat. Collected from a live *Spirostreptus* sp. (from India) in Hamburg.

Hypoaspis (*Coleolaelaps*) *amazon* Berl. 1920

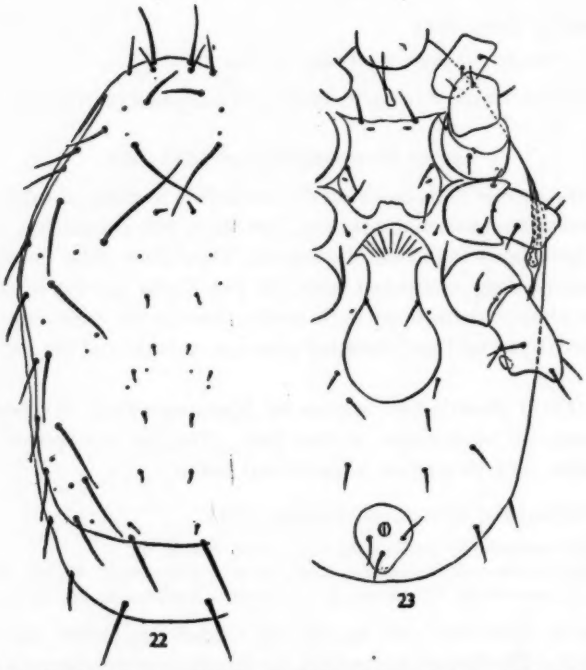
1920. *Coleolaelaps amazon*, Berlese, A., *Redia*, **14**, 156.

This species may be distinguished from other Hypoaspid paraphages of Myriapoda by the presence of a pair of long whip-like setae situated posteriorly on the dorsal shield.

Habitat. On *Scolopendra* sp., Italian Somaliland (Bardeira).

Hypoaspis polydesmoides n.sp.

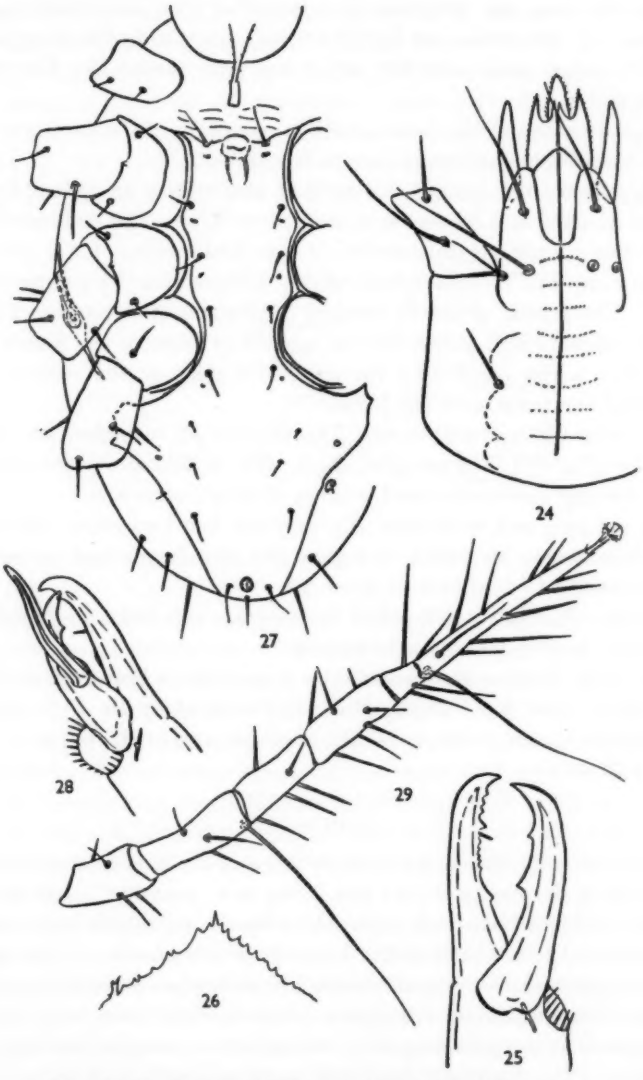
Female. The dorsal shield (1400 × 825 μ) is truncated posteriorly and bears twenty-eight pairs of simple setae of varying lengths (Fig. 22). Seven pairs situated mid-dorsally are considerably shorter than the others. The distribution of setae and 'pores' is shown in the figure. The dorsal shield does not completely cover the dorsum of the mite.



Figs. 22–23. *Hypoaspis* (*Hypoaspis*) *polydesmoides* n.sp. Female.
Fig. 22. Dorsal view. Fig. 23. Ventral view.

Ventrally, the tritosternum is well-developed with long ciliated lacinae (Fig. 23). The sternal shield, truncated anteriorly and incised posteriorly extends from the anterior margin of coxae II to the middle of coxae III. It carries three pairs of simple setae and two pairs of 'pores'. The metasternal setae are short and lie on the interscutal membrane in line with the posterior margin of coxae III. The third pair of 'pores' is situated anterior to the metasternals. The genital shield is flask-shaped and extends beyond the posterior margins of coxae IV by more than half its length. It has one pair of setae. The hyaline expansion of the dorsal shield is well developed and extends beyond the level of the posterior margin of coxa III. The pear-shaped anal shield with three setae is distant from the genital shield. The striated membrane of the postero-ventral region is provided with four to five pairs of simple setae. The metapodalia are small.

The stigmata are situated ventro-laterally between coxae III and IV. The peritreme and peritrematal plate are well developed, the former extending beyond coxa I.



Figs. 24–29. *Hypoaspis* (*Hypoaspis*) *polydesmoides* n.sp.

Fig. 24. Gnathosoma of female (ventral). Fig. 25. Chelicera of female. Fig. 26. Tectum of female. Fig. 27. Ventral of male. Fig. 28. Chelicera of male. Fig. 29. Leg IV of male.

The gnathosoma bears the normal four pairs of setae ventrally. The internal posterior rostrals and the rostrals are twice the length of the other two pairs (Fig. 24). The corniculi are long and slender. The ventral groove is provided with

seven rows of denticles. The chelicerae are strongly developed, the fixed digit is multidentate and has a short stout *pilus dentilis* (Fig. 25). The movable digit is bidentate. The tectum is triangular in shape with denticulated margins (Fig. 26). All the legs are long and terminate in a pulvillus with two claws: leg I 1430μ, leg II 1030μ, leg III 1050μ and leg IV 1600μ. Long whip-like setae are present on femur II, femur and genu III and femur and tarsus IV. The females are ovoviviparous.

Male. The structure of the dorsal shield is essentially the same as in the female. The shield completely covers the dorsum in this sex.

Ventrally, the sternal, genital, ventral and anal shields are fused (Fig. 27). The distribution of setae and 'pores' is shown in the figure. The endo-podals are also fused with the sterniti-genital portion of the shield except for a short distance posterior to coxae IV. The chaetotaxy of the 'ventral' shield posterior to coxae IV is variable. The genital orifice is situated on the anterior margin of the sternal shield. The stigmata and peritremes are similar to those in the female. The peritrematal plate is not fused with the exo-podal plate or the ventral plate. The gnathosoma is the same as in the female.

The chelicerae are well sclerotized. The fixed digit is unidentate and bears a *pilus dentilis* (Fig. 28). The movable digit, also unidentate, is provided with an anteriorly directed spermatophoral process of simple structure.

The legs are long and terminate in a pulvillus and two claws. Whip-like setae are present on femur II, femur and genu III, and femur and tarsus IV. Their distribution on leg IV is shown in figure 29.

Dimensions. Female: 1500–1650μ in length, 830–860μ in breadth. Male: 1250–1300μ in length, 600–625μ in breadth.

Locality. Four females and three males from a giant Polydesmid (Diplopoda); Fraser's Hill (*c.* 3000 ft.), Pahang, Malaya. Collected by Dr J. R. Audy, 8 July 1948. Holotype female (1954:6:14:31), allotype male (1945:6:14:32), paratypes (1954:6:14:33–37).

DISCUSSION

The species dealt with above demonstrate some of the modifications certain structures undergo in the change from a free-living to a 'parasitic' mode of life. In the paraphages of Myriapoda and other Arthropods reduction may occur in the sclerotization and chaetotaxy of the body, dentition and size of the digits of the chelicerae, chaetotaxy of the gnathosoma and pedipalps, peritreme and peritrematal plate and the ambulacral apparatus. These modifications, however, are by no means restricted to the paraphages but are apparent to a greater or lesser extent in the majority of the Laelaptid ecto- and endo-parasites of mammals, birds and reptiles. The endo-parasites also show a marked reduction or absence of the bipartite tritosternum.

Vitzthum (1942), discussing the phylogeny of the Laelaptidae s.lat. suggested that the Hypoaspidinae are the most primitive group and that they have given rise on the one hand to the heavily sclerotized Laelaptinae and, on the other, to the poorly sclerotized forms of the Liponyssinae (cf. Fig. 30). The writer finds him-

self in agreement with this statement. The *Hypoaspis*-group has many qualities which support this hypothesis. It comprises a generalized group of mites which has become established in a variety of terrestrial habitats and has a wide range of geographical distribution. In its non-specialized form, and one can take *H. polydesmoides* figured above as an example, the legs are well-developed and provided with an ambulacral apparatus comprising a pulvillus and two claws, the body is well sclerotized and the chelicerae are chelate-dentate. The free-living members of the group which are predominantly predatory in habit, tend to show increased sclerotization of the ventral surface. It is probable that the heavily armoured parasitic species of the Laelaptinae were derived from them. On the other hand,

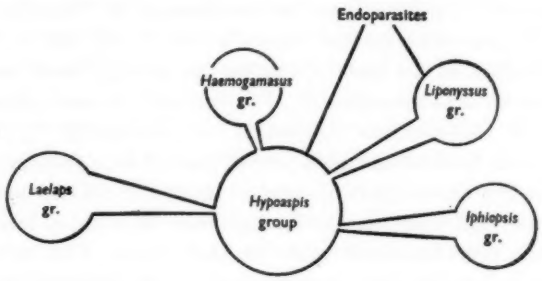


Fig. 30. A diagrammatic representation of the phylogeny of the Laelaptidae.

the *Hypoaspis*-group contains a number of forms which show a tendency towards 'parasitism' either as paraphages of Arthropods, e.g. *Julolaelaps* and *Hypoaspis* spp., or as ectoparasites of mammals, e.g. *Haemolaelaps*. The transition from a free-living to a 'parasitic' mode of life often shows little or no change in external morphology, and makes the separation of these groups on morphological grounds alone impossible. The extreme specialization exhibited by the endo-parasitic species of the Laelaptidae obscures their relationship with other members of the family. It is probable that some genera, e.g. *Entonyssus* have arisen from the Liponyssid-type, whilst others have developed independently from the primitive *Hypoaspis*-group.

To attempt a phylogenetic classification of the Laelaptidae in the present state of our knowledge of the family is virtually impossible. Within a family showing such diversity of form it seems advisable to concentrate on a practical classification emphasizing differences rather than resemblances between groups of genera.

The classification given by Vitzthum (1943) in which the Laelaptidae was divided into thirteen subfamilies is, as Zumpt & Patterson (1951) have said, 'a better fit for practical purposes' than to split the Laelaptidae into a number of families. Vitzthum's concept of the subfamilial divisions of the family, however, requires modification. The type genus of the Podocininae is closely related to the Macrochelid-group and should be transferred to the Parasitoidea as Evans (1954) has suggested. The writer also considers that the Phytoseiinae comprise a natural group distinct from the Laelaptidae and worthy of family status. The Phytoseiinae

sensu Vitzthum and the majority of the remaining genera in the Podocininae should be transferred to the Phytoseiidae. This leaves the following eleven subfamilies which will be discussed below:

(1) Laelaptinae Tragardh, 1908
(2) Hypoaspidinae Vitzthum, 1940
(3) Haemogamasinae Oudms., 1926
(4) Iphiopsinae Kramer, 1886
(5) Liponyssinae Ewing, 1923
(6) Dermanyssinae Kolenati, 1859
(7) Raillietiinae Vitzthum, 1942
(8) Eviphidinae Berl., 1913 (syn. *Hyletastinae* Vitzthum, 1942)
(9) Entonyssinae Ewing, 1923
(10) Rhinonyssinae Trouess., 1895
(11) Halarachninae Oudms., 1906

The two subfamilies containing the majority of the non-specialized Laelaptids, the Laelaptinae and Hypoaspidinae, were separated by Vitzthum chiefly on the chaetotaxy of the genital or genital-ventral shield in the female. This separation of the two subfamilies on the basis of whether the genital shield bears one or more than one pair of setae is not practicable. For example, in some species of the genera *Haemolaelaps* and *Androlaelaps* (placed in the subfamily Hypoaspidinae and characterized by the females having a pair of genital setae) the genital shield may carry one, two or even three pairs of setae so that some species would be placed in the Hypoaspidinae and others in the Laelaptinae. In view of this the writer proposes to unite the Hypoaspidinae with the Laelaptinae. This removes the major stumbling block in any attempt on a practical classification of the Laelaptidae.

The Haemogamasinae which has been revised recently by Keegan (1951) comprises a small group of mites parasitic on small mammals and birds. This subfamily is distinguished from the Laelaptinae chiefly by the increased chaetotaxy of the dorsal and ventral plates, and the inter-scutal membrane, and by the presence of an undivided dorsal shield in the nymphal stages. Dense setation of the dorsal shield and the interscutal membrane also occurs in some *Hypoaspis* species parasitic on mammals, and in the genus *Eulaelaps* Berl. the genital shield is also affected. In these genera, however, the dorsal shield in the nymphal stages is divided.

The Iphiopsinae include the paraphages of arthropods. They are characterized by the reduction of the peritreme and usually of the claws of one or more pairs of legs. There seems little doubt that these paraphages arose from the *Hypoaspis*-group. Many species are ovoviviparous or viviparous as are some of the less specialized paraphages in the genus *Hypoaspis* s.lat. The Iphiopsinae comprises the following genera: *Iphiopsis* Berl., *Jacobsonia* Berl., *Scolopendracarus* Evans, *Berlesia* Can., *Dinogamasus* Kramer and *Dynatochela* Keegan.

The Liponyssinae and Dermanyssinae may be considered together since they contain the majority of the specialized ecto-parasites of mammals, birds and reptiles. The chelicerae in the female lack teeth and the body is usually poorly sclerotized. The subfamilies may be distinguished by the structure of the chelicerae which are shear-like in the Liponyssinae and stylet-like in the Dermanyssinae.

The Raillietiinae was erected by Vitzthum (1942) for the genus *Raillietia* Trouess. which contains two species only. Both are parasites of the external auditory meatus of the *Bovoidea*. The body is weakly sclerotized and the chelicerae

in the female are shear-like. Vitzthum considered the Raillietiinae to differ from the Liponyssinae in the absence of a tritosternum in both sexes ('kein tritosternum'). The writer has examined specimens of the two known species of *Raillietia* and found the tritosternum to be well developed and bipartite. It is possible that Vitzthum did not examine specimens of the genus but relied on descriptions and figures by other workers for his definition. The writer, therefore, transfers *Raillietia* to the Liponyssinae and relegates the subfamily Raillietiinae to the synonymy.

The remaining four subfamilies are less clearly defined than those considered above. A considerable amount of revisionary work is necessary before one can attempt to discuss their relationships with other members of the family.

The Eviphidinae comprises a heterogeneous group of genera which is usually characterized by the structure of the tectum. The ventral plating in the male differs considerably from that in the Laelaptinae in that the anal shield is separate from a sterniti-genital shield. The subfamily contains both free-living species and paraphages.

The subfamilies containing the endoparasitic forms, namely, the Entonyssinae, Halarachninae and Rhinonyssinae, all have the tritosternum markedly reduced or absent. In many instances there is strong morphological similarity between genera or groups of genera, especially in degree of sclerotization of the body, structure of the chelicerae and the peritreme. These characters, being adaptive to a habitat, may be convergent. This complicates the study of their phylogeny. At present the three subfamilies are separated chiefly on ecological rather than morphological grounds.

The nine subfamilies included by the writer in the Laelaptidae may be distinguished as follows. It must be emphasized, however, that the classification is predominantly a practical one. It will undoubtedly be greatly modified as more detailed studies are carried out on chaetotaxy and post-embryonic development within the family.

A KEY TO THE SUBFAMILIES OF THE LAELAPTIDAE BERL. 1892

1. A bipartite tritosternum present in both sexes; free-living, paraphages or ecto-parasites. **2**

— Without a bipartite tritosternum in both sexes; endo-parasites showing extreme specialization. **7**

2. Chelicerae in the female with teeth (except in *Dinogamasus*, in which the fixed digit of the chelicera is markedly reduced in size, and in some species of *Haemogamasus**); fixed digit usually with *pilus dentilis*. Dorsal and ventral shields well-developed and strongly sclerotized except in some paraphages of arthropods. **3**

— Chelicerae in female without teeth; fixed digit often without *pilus dentilis*. Dorsal and ventral shields usually reduced or poorly sclerotized. **6**

3. Peritreme and peritrematal plate well developed in both sexes and extending up to or beyond the level of coxa I. **4**

— Peritreme markedly reduced or absent; never reaching beyond the anterior margin of coxa III. **Iphiopsinae** Berl.

* The species of *Haemogamasus* lacking teeth on the chelicerae may be readily separated from the Liponyssinae and Dermanyssinae by the increased setation of the ventral shields.

4. Dorsal and ventral shields, and inter-scutal membrane densely clothed with setae. Sternal shield often with more than three pairs of setae. Dorsal shield entire in nymphal stages. **Haemogamasinae** Oudms.

— Dorsal and ventral shields with a moderate covering of setae. Sternal shield with not more than three pairs of setae. Dorsal shield divided in nymphal stages. 5

5. Tectum elongate, lance-like in both sexes. Male with sterniti-genital shield separate from anal shield. Free-living or paraphages. **Eviphidinae** Berl.

— Tectum not lance-like, usually convex with a serrated margin. Male with sterniti-genital shield fused with ventral or ventri-anal shield. Free-living, paraphages or ecto-parasites. **Laelaptinae** Tragardh.

6. Chelicerae shear-like in female. **Liponyssinae** Ewing.

— Chelicerae stylet-like in female. **Dermanyssinae** Kolenati.

7. Parasites of respiratory tract of mammals; genital plate rudimentary or absent; palptibia and tarsus fused. **Halarachninae** Oudms.

— Parasites of the respiratory tract of reptiles; genital shield present with a pair of setae; dorsal shield entire. **Entonyssinae** Ewing.

— Parasites of the respiratory tract of birds; genital shield present with or without setae; dorsal shield entire, divided or absent. **Rhinonyssinae** Trouess.

SUMMARY

1. A review is given of the Laelaptid paraphages of the Myriapoda with a key to the genera.

2. One new genus, *Scolopendracarus* (type: *S. brevipilis*) and a new subgenus, *Parajacobsonia* (type: *Jacobsonia tertia* Vitzthum) of the genus *Jacobsonia* Berl. are proposed.

3. The following three species are described as new to science: *Jacobsonia* (*Jacobsonia*) *audyi*, *Scolopendracarus brevipilis*, *Hypoaspis* (*Hypoaspis*) *polydesmoides*.

4. The phylogeny and classification of the family Laelaptidae Berl. are discussed. A key is given to the subfamilies of the Laelaptidae.

The writer is grateful to Dr J. R. Audy, Malaya, for the specimens of two new species described in this paper, and to Dr H. W. Parker for his criticism of the manuscript.

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DIROFILARIA AETHIOPS WEBBER, 1955, A FILARIAL PARASITE OF MONKEYS

I. THE MORPHOLOGY OF THE ADULT WORMS AND MICROFILARIAE

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National Institute for Medical Research, London

(With 12 Figures in the Text)

A filarial worm belonging to the genus *Dirofilaria* Railliet & Henry, 1911 has been found in African green monkeys (*Cercopithecus aethiops*). The adult worms live in the interfascicular connective tissue and are especially common between the muscles of the thigh. The microfilariae are found in the blood and exhibit nocturnal periodicity. For several years the infection has been maintained in our laboratory for experimental purposes. In the present paper a description of *D. aethiops* Webber, 1955 is given, followed by a brief redescription of *D. schoutedeni* Vuylsteke & Rodhain, 1938, to which *D. aethiops* is closely related. Subsequent papers will deal with transmission in the laboratory and the life history of *D. aethiops*, including the larval stages in the intermediate host.

Dirofilaria aethiops

The worms were found lying singly or in small groups in the fascial tissue on the surface of the muscle bundles, both under the skin and between the muscles. Worms were recovered from the following naturally infected monkeys which came from both East and West Africa.

(1) *Cercopithecus aethiops johnstoni* (monkey 185), from Tinde, Tanganyika. Two female, three male worms.

(2) *C. a. johnstoni* (monkey 190), from Entebbe, Uganda. One female, three male worms.

(3) *C. a. johnstoni* (monkey 192), from Entebbe, Uganda. Three female, one male worms.

(4) (Probably) *Colobus badius temmincki* (monkey A), from Kenaba, Gambia. One male worm.

(5) *Cercopithecus* sp. (monkey X). One female, one male worm.*

In addition, worms from five monkeys which had been infected in the laboratory were available for study. The dimensions of the worms are shown in Table 1. The worms were cylindrical, tapering towards the head and tail. The cuticle was smooth, but there were transverse striations on the ventral surface of the tail of the male. The females were 163–225 mm. long. The vulva was oesophageal or just posterior to the oesophagus, which was 1·7–2·0 mm. long. The intestine was narrow and

* These worms were kindly lent by Professor J. J. C. Buckley of the London School of Hygiene and Tropical Medicine.

the anus was inconspicuous. The tail was 70μ long and had a terminal phasmid. The males were 46–81 mm. in length and the tail end was spirally coiled. The oesophagus was 1·2–1·7 mm. long and the cloacal aperture was 0·1–0·15 mm. from the posterior extremity. The caudal alae did not encircle the rounded end of the tail. The left spicule was 400–630μ long and the right spicule 200–260μ. The left spicule (Figs. 1 and 2) was divided into three regions. The proximal region was cylindrical with 'thickened' walls. The mid-region was as wide as the proximal

Table 1. *Dimensions of* Dirofilaria schoutedeni *and* D. aethiops

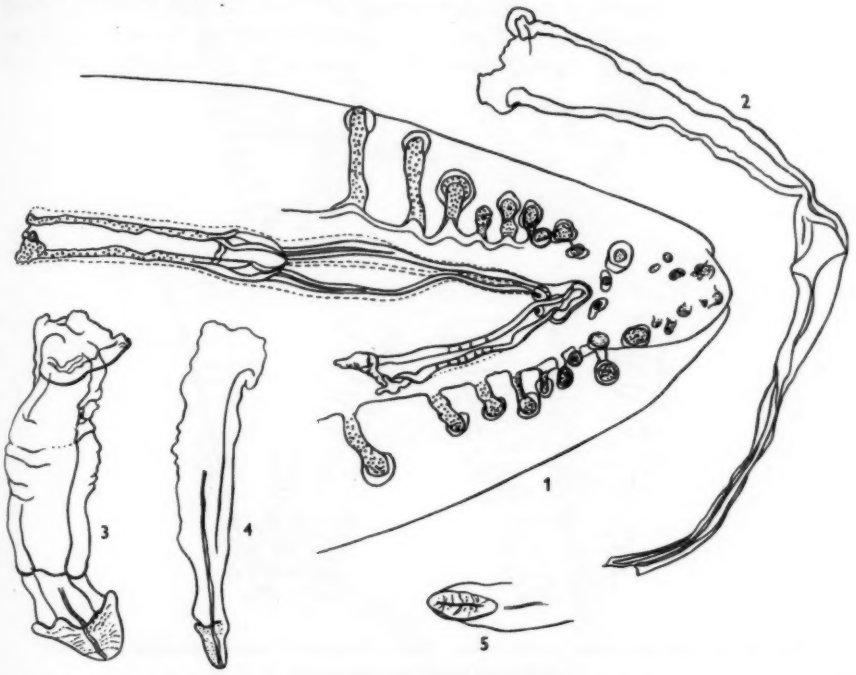
	D. schoutedeni Vuylsteke & Rodhain (1938)	*D. aethiops* Author's figures
Female		
Length (mm.)	270–295	163–225
Maximum width (μ)	600–630	573–710
Distance of nerve ring from anterior (μ)	—	266–342
Length of oesophagus (μ)	1640–1730	1700–1980
Distance of vulva from anterior (μ)	2890–3700	1600–2090
Length of tail (μ)	70	70
Male		
Length (mm.)	86	46–81
Maximum width (μ)	360	270–489
Distance of nerve ring from anterior (μ)	—	254–320
Length of oesophagus (μ)	1230	1220–1690
Length of tail (μ)	98	109–151
Length of left spicule (μ)	448	400–630
Length of right spicule (μ)	190 (V. & R.) 261 (W.A.F.W.)	200–260
Spicular ratio	2·4:1 (V. & R.) 1·7:1 (W.A.F.W.)	1·7–2·7:1
No. of pairs of pre-anal papillae	4–6 large 1 small	6–8
No. of pairs ad- and post-anal papillae	1 large 7–8 small	9

region, but the thickening was less. At the junction of the proximal and mid-regions there were small supporting rods (Fig. 1), in addition to the thickened walls of the spicule. The distal region of the spicule tapered and at the tip two slender, pointed rods supported a delicate membrane (Fig. 2).

The right spicule (Figs. 3–5) was thickened and cylindrical. In the proximal part of the spicule there were transverse ridges (Fig. 3). The distal part of the spicule narrowed slightly and ended in a cap-like structure, which was best seen when the spicule was gently flattened.

The caudal papillae (Figs. 1 and 6) were bilaterally symmetrical in arrangement. There were six pairs of large, club-shaped papillae in front of the cloacal aperture, 1 to 3 pairs lateral to the aperture, and 1 pair behind the aperture. On the anterior border of the cloacal aperture there was occasionally a pair of papillae, but more often these were represented by a single prominence and even this was sometimes absent.

There were six pairs of small papillae behind the cloacal aperture. The papillae of the most posterior pair often had a square end and the papillae immediately in front of these tapered to a fine point at the surface of the caudal alae. The remaining four pairs of small papillae were usually rounded, but one or more pairs were sometimes pointed.



Figs. 1–5. *Dirofilaria aethiops*, male from monkey A. 1, tail, ventral view, × 250; 2, left spicule, × 400; 3, right spicule, ventral view, × 400; 4, right spicule, dorso-ventral view, × 700; 5, right spicule, tip, lateral view, × 400.

Microfilaria

The microfilaria of *D. aethiops* (Fig. 7) is found in the blood. The dimensions of the microfilariae measured in stained blood smears from the different monkeys are shown in Table 2.

The microfilariae were unsheathed. A nerve ring, an excretory pore and an anal pore were visible as gaps in the nuclear column. The head was usually rounded, but a few specimens were fixed with the tip withdrawn leaving a squared outline. The tail was long and tapered and ended in a narrow filament.

Dirofilaria schoutedeni

Two females and one male worm were described by Vuylsteke & Rodhain (1938). The worms were found in the subcutaneous connective tissue of a monkey (*Colobus polykomos uelensis*) from Manda, Uélé, Belgian Congo. The dimensions are shown

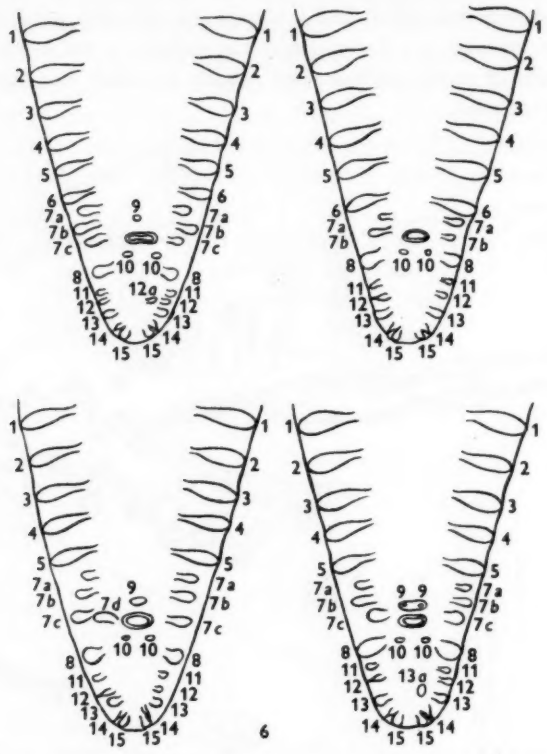


Fig. 6. *D. aethiops* from monkey 185. Male, diagram of caudal papillae, showing variation between specimens.

Table 2. *Dimensions of* Dirofilaria aethiops *microfilariae*

Monkey no.	No. of micro-filariae measured	Length (μ)	1st.n.	n.r.	ex.p.	a.p.	l.n.
185	18	201 ± 13	2·5 ± 0·5	18·8 ± 1·8	27·6 ± 1·7	—	10·8 ± 1·9
190	13	195 ± 18	2·5 ± 0·5	20·9 ± 1·6	31·1 ± 1·9	78·1 ± 2·8	5·7 ± 1·9
X	6	200 ± 24	3·1 ± 0·6	20·1 ± 1·3	31·0 ± 2·2	77·0 ± 3·2	7·5 ± 2·1
151	13	240 ± 38	2·3 ± 0·5	20·5 ± 1·1	32·5 ± 2·6	80·0 ± 1·9	3·4 ± 1·0
163	15	181 ± 6	2·5 ± 0·4	21·6 ± 1·3	31·6 ± 1·5	78·3 ± 1·9	4·4 ± 1·0
191	12	186 ± 23	2·2 ± 0·4	21·2 ± 1·6	32·5 ± 1·5	76·1 ± 4·6	7·9 ± 4·9
192	12	198 ± 13	2·7 ± 0·4	20·7 ± 3·0	31·9 ± 3·2	76·9 ± 2·2	5·9 ± 1·6
B	13	219 ± 6	3·4 ± 0·4	18·6 ± 1·2	29·7 ± 1·7	77·6 ± 1·3	6·7 ± 1·3
D	13	222 ± 13	2·95 ± 0·5	19·6 ± 1·0	33·9 ± 2·6	78·6 ± 1·6	4·4 ± 0·9

Abbreviations. Length: Total length microfilaria in μ excluding sheath; 1st n.: % length from anterior extremity to first nucleus of nuclear column; n.r.: % length from anterior end to nerve ring; ex.p.: % length from anterior end to excretory pore; a.p.: % length from anterior end to anal pore; l.n.: % length from posterior end to last nucleus of nuclear column.

in Table 1. The worms were kindly lent by Dr Darteville of the Musée du Congo Belge, and examination revealed the following details of the spicular morphology and caudal papillae:

The left spicule (Fig. 8) is cylindrical in the proximal part with 'thickened' walls. About $\frac{1}{2}$–$\frac{2}{3}$ of the way along it shows the twist characteristic of the genus *Dirofilaria*. Distally it is much more slender and ends in a fine point. The thickened cylindrical part of the spicule, proximal to the twist, is more than half its length. The short spicule (Fig. 9) is wider and more strongly thickened than the long

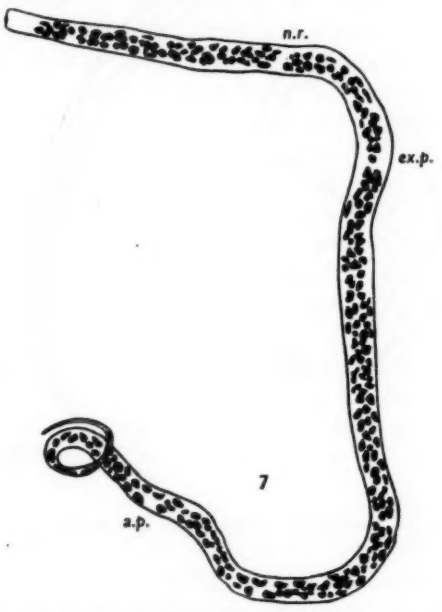
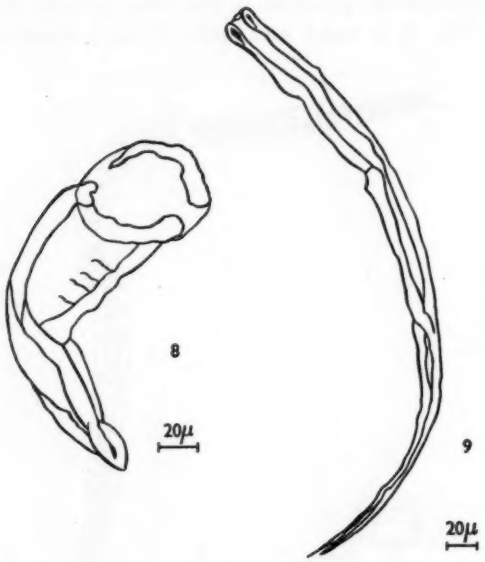


Fig. 7. *D. aethiops* microfilaria from monkey 185.

spicule. In the proximal part of the spicule there are transverse ridges and in the distal part there are longitudinal markings. There is a small membranous portion at the extreme distal tip of the spicule. The caudal papillae (Fig. 10) are nearly bilaterally symmetrical. There are large club-shaped papillae extending from well in front of the cloaca to a short distance behind it, and also small papillae between the cloaca and the tip of the tail. One 'pair' of papillae is represented by a slight bump above the surface of the cuticle on the anterior border of the cloaca.

The adult worms of *D. schoutedeni* and *D. aethiops* are similar to each other, but they differ in the cuticular ornamentation and the spicular morphology. The microfilaria of *D. schoutedeni* has not been described. The cuticle of the ventral surface of the tail of the male of *D. schoutedeni* is ornamented by slight prominences which extend as far forwards as the spiral coiling of the worm, but in *D. aethiops* there is no ornamentation except for transverse striations. In the left spicule the 'twist' is nearer the proximal end in *D. aethiops* and nearer the distal end in

D. schoutedeni. In *D. aethiops* the pattern of supporting rods at the twist is more complex than in *D. schoutedeni*. In the right spicule *D. aethiops* lacks the longitudinal markings found in the distal part in *D. schoutedeni*. In *D. aethiops* the distal end is capped, but not in *D. schoutedeni*. These characters were constant in all the males of *D. aethiops* which were examined.



Figs. 8, 9. *D. schoutedeni*, male: 8 left spicule; 9, right spicule.

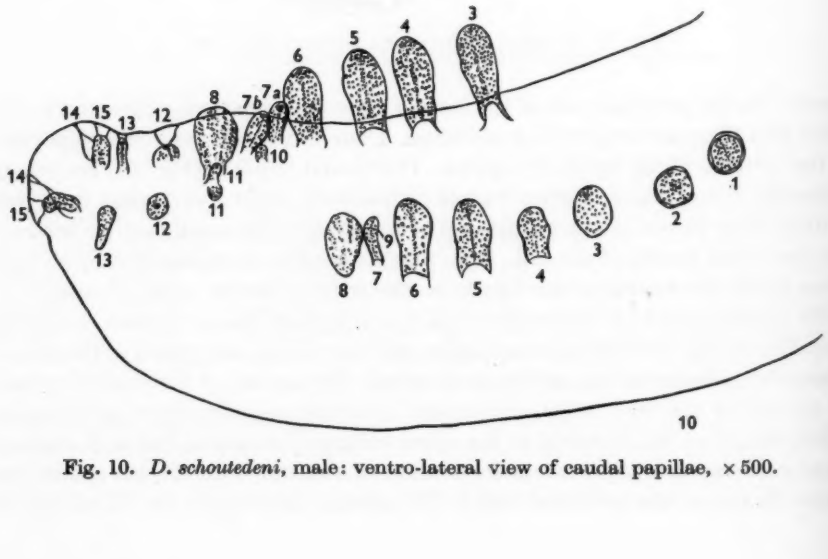


Fig. 10. *D. schoutedeni*, male: ventro-lateral view of caudal papillae, × 500.

Geographical distribution

As stated above (p. 369), the monkeys infected with *D. aethiops* came from different parts of Africa—one from Tanganyika, two from Uganda and one from Gambia. In addition, there were several monkeys from which there was no opportunity of recovering adult worms, but which contained microfilariae which were indistinguishable from those of *D. aethiops*. These latter monkeys came from different places, as follows:

(1) *Cercopithecus aethiops sabaeus* (monkey 151), from Gambia.
(2) *C. a. johnstoni* (monkey 163), from Taveta, Kenya.
(3) *C. a. johnstoni* (monkey 164), from Tinde, Tanganyika.
(4) *C. a. johnstoni* (monkey 191), from Entebbe, Uganda.
(5 and 6) (Probably) *Colobus badius temmincki*, (monkeys B and D), from Kenaba, Gambia.

The microfilariae in these monkeys almost certainly belonged to *Dirofilaria* and may have been *D. aethiops*. They provide evidence of the broad geographical distribution of the genus in Africa, which was also shown by the monkeys in which adult *D. aethiops* and *D. schoutedeni* were found. The monkeys infected with *D. aethiops*, both *Cercopithecus* and the Gambian monkeys, occurred in savannah bush country.

Microfilariae which probably belonged to *Dirofilaria* have been reported previously from Uganda (Low, 1904; Nabarro & Greig, 1905); Nyasaland (Sleeping Sickness Commission of the Royal Society, 1915); Belgian Congo (Broden, 1919); and French Guinea (Leger, 1922).

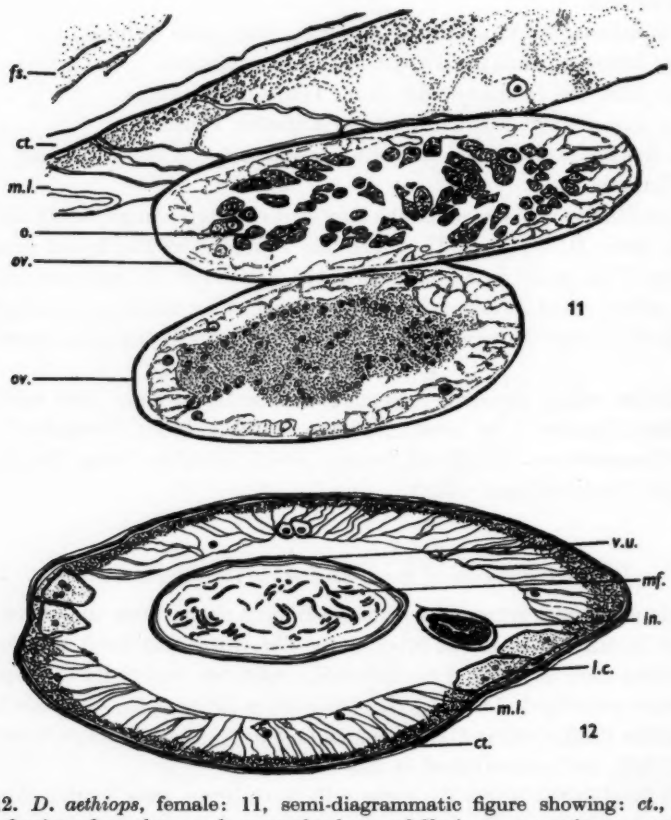
Internal structure of the adult female Dirofilaria aethiops

A female worm was removed with the adjacent tissue from one of the monkeys which had been infected in the laboratory. The worm was fixed in formol saline, serial sections were made, and the internal structures, especially the reproductive system, were examined. The reproductive system is didelphic and opisthodelphic, and resembles that of other filariae, e.g. *Litomosoides carinii* as described by Cross & Scott (1947), and also studied in this laboratory.

At each level in the worm the germ cells or embryos were further developed in one germinal tube than in the other. Fig. 11 shows part of a section passing through the worm and the host tissue. In the upper left corner of the figure is a strand of the connective tissue of the monkey, below this is the body wall of the worm, and the remainder of the figure consists of a section through each ovary. The lower section passes through the germinal zone of one ovary and the upper section through the growth zone of the other ovary. The growth zone contains oogonia which were attached to a central rachis (not cut in the section shown). The wall of the ovary appears to be thick, in contrast to its appearance in fresh material as a thin transparent membrane; the thickness of the wall in the sections may have been due, at least in part, to changes which had occurred during fixation. The oviduct was seen in the sections as a thick-walled region of the germinal tube; it was narrower than

the ovary or uterus and contained few developing cells. There was no distinct spermatheca, but spermatozoa were found in a mass in the distal part of each uterus.

Development from ova to microfilariae takes place as the embryos move forward in the uteri towards the anterior end of the worm. In living material observed on one occasion microfilariae which were not quite fully developed were seen moving



Figs. 11, 12. *D. aethiops*, female: 11, semi-diagrammatic figure showing: *ct.*, cuticle of filaria; *fs.*, fasciae of monkey; *m.l.*, muscular layer of filaria; *o.*, oogonia; *ov.*, ovary, × 300. 12, section showing: *ct.*, cuticle; *in.*, intestine; *l.c.*, lateral cord; *mf.*, microfilariae; *m.l.*, muscular layer, *v.u.*, vagina uterina. × 150.

actively inside the egg membranes. The microfilariae hatched in the proximal part of the uterus or in the vagina uterina. In prepared sections of the worm the empty egg membranes could not be seen (although they had been observed in fresh material mounted in a saline solution), but unhatched microfilariae were coiled inside the membranes while those which had hatched were uncoiled and extended. Fig. 12 shows a complete section of the worm in the region of the vagina uterina and in this organ nearly all the microfilariae were uncoiled. Fig. 12 also shows the

intestine, the large lateral cords, and the muscles of the body wall which are divided by the lateral cords into dorsal and ventral halves.

The vagina vera was a short, twisted tube lined with cuticle, surrounded by muscles and opening on the surface of the worm by the slit-like vulva.

SUMMARY

1. *Dirofilaria aethiops* Webber, 1955 has been discovered in African monkeys (*Cercopithecus aethiops*). Adult worms were found in the interfascicular connective tissue, especially in the thighs; microfilariae were found in the blood.

2. The adult worms are morphologically similar to other members of the genus, from which they differ principally in the structure of the spicules of the male worms. The thickening of the left spicule is complex at the twist; the latter is nearer to the proximal than to the distal end. The right spicule is robust and ends in a cap-like structure.

3. The microfilariae are indistinguishable from those of other members of the genus.

4. In *Dirofilaria schoutedeni* Vuylsteke & Rodhain, 1938 the left spicule is simpler in structure than the left spicule of *D. aethiops*, and the twist is nearer to the distal end than to the proximal end. The distal part of the right spicule of *D. schoutedeni* has longitudinal markings but no cap.

5. *D. aethiops* has been recorded from Tanganyika, Uganda and Gambia, in *Cercopithecus* and *Colobus* monkeys.

6. The anatomy of the female *D. aethiops*, as it is shown in serial sections, is briefly described.

I wish to thank Dr F. Hawking for advice and encouragement and Mr F. Higginson for the preparation of serial sections.

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DIROFILARIA AETHIOPS WEBBER, 1955, A FILARIAL PARASITE OF MONKEYS

II. MAINTENANCE IN THE LABORATORY

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(With 1 Figure in the Text)

The first paper of this series (Webber, 1955*b*) reported that *Dirofilaria aethiops* Webber, (1955*a*) is a filarial worm of African green monkeys (*Cercopithecus aethiops*). This paper describes its maintenance in the laboratory and the course of the infection in the vertebrate host. Five monkeys with natural infections were obtained from Africa: three from Entebbe, Uganda, one from Gambia, and one from Tinde, Tanganyika Territory. As described in the text below, the strain was transmitted to nine other monkeys.

PERIODICITY OF THE MICROFILARIA

The periodicity of the microfilariae in the peripheral circulation was demonstrated by taking two 20 mm.^{3} samples of blood from infected monkeys every 2 hr. for a period of 30 hr. The blood films were dried, dehaemoglobinized, fixed, and stained with haemalum or Giemsa's stain, and the number of microfilariae was counted. The accuracy of the microfilaria counts as a measure of the number contained in the circulating blood has been discussed in detail by Galliard & Ngu (1947). The principal sources of error involved are as follows:

(*a*) The size of the sample is small compared with the total volume of the blood in the body.

(*b*) The number of microfilariae in the blood is not constant throughout the circulatory system, but varies from organ to organ, and between the arteries, veins and capillaries of the same organ. This is probably the chief source of variation.

(*c*) The technique of fixing, staining and counting may vary, but with proper care this should be a minor source of variation.

The results of the counts from monkeys 188 and 197 are shown graphically in Fig. 1, and similar curves were obtained from the other monkeys. The microfilariae have a strongly marked nocturnal periodicity similar to that of *Microfilaria bancrofti*. The variations outlined above do not obscure the main characteristics of the microfilarial cycle, but they may account for the large fluctuations which occur during the nocturnal rise and fall. The maximum number of microfilariae was found between 11 p.m. and 1 a.m. and the minimum between 10 a.m. and 2 p.m. The minimum count was rarely more than 1 % of the maximum.

TRANSMISSION OF INFECTION

The development of *Dirofilaria aethiops* in mosquitoes is described in detail in the third paper of this series (Webber, 1955*c*). Development proceeded moderately well in *Aëdes aegypti* and less well in *Anopheles maculipennis* var. *atroparvus*; all the monkeys, therefore, were inoculated from *Aëdes aegypti*. The mosquitoes were suspended in saline solution and the suspension was injected subcutaneously, or inserted into a subcutaneous pocket made under anaesthesia. The method of

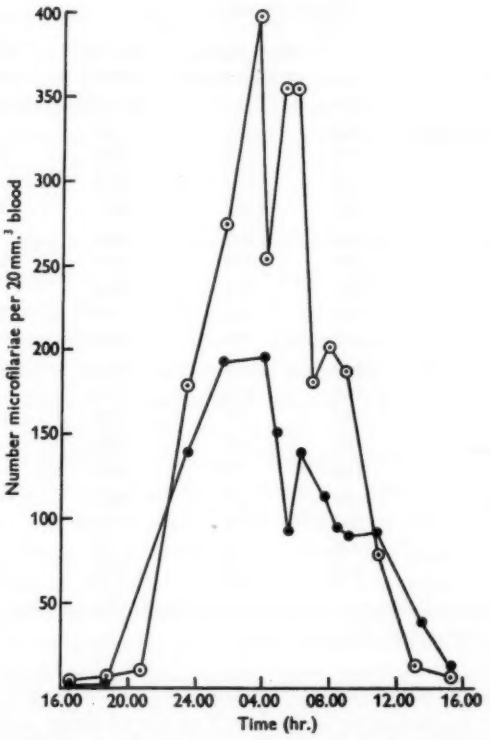

Fig. 1. Chart to show nocturnal periodicity exhibited by *Dirofilaria aethiops* in blood of monkeys. ⊙—⊙, monkey 188, *Cercopithecus aethiops*; ●—●, monkey 197, *Macaca mulatta*.

inoculating a suspension of mosquitoes had previously been used with *Dirofilaria repens* and *D. immitis* in dogs, and had the advantage that all the available infective larvae were put into the monkey. If the mosquitoes had been allowed to feed on the monkey, only some of the larvae would have left the mosquito and perhaps not all of these would have penetrated the skin. In view, however, of the many failures to produce infection it is possible that some modification of the technique might have been advantageous, e.g. removal of the worms from the mosquito by dissection.

Fourteen monkeys were inoculated with batches of mosquitoes suspended in

saline solution. Each batch of mosquitoes was divided among several monkeys, and each monkey received worms from more than one batch of mosquitoes. On three occasions abscesses developed at the site of the injection, but they cleared up in a few days. The monkeys were five *Cercopithecus aethiops johnstoni*, six *C. a. sabaeus*, one *Cebus* sp., one *Cercocebus* sp. and one *Macaca mulatta*.

Table 1. *Transmission experiments with* Dirofilaria aethiops

Recipient monkey	Donor monkey: No.	Donor monkey: Microfilariae per 20 mm.³ blood	No. of *Aëdes* injected	No. of worms injected	Interval between inoculation and finding microfilariae (weeks)
164. *Cercopithecus aethiops johnstoni*	185	581	25	8	21
	185	218	200	120	11
187. *C. a. johnstoni*	185	710	400	80	47
	185	1003	400	240	42
188. *C. a. johnstoni*	185	710	400	80	47
	185	1003	400	240	42
189. *C. a. johnstoni*	185	710	400	80	47
	185	69	75	150	41
	185	50	250	60	11
194. *C. a. sabeus*	185	1647	400	680	52
	185	488	100	20	47
	185	77	300	1100	22
197. *Macaca mulatta*	185	191	300	210	51
	185	75	250	75	50
175. *C. a. johnstoni*	164	40	300	60	None found
	185	575	70	70	None found
	185	78	40	10	None found
	185	216	500	250	None found

Note: column 3. The microfilaria count is the average of two counts, one taken before and one taken after the mosquitoes fed.

Table 1 gives the details of the seven monkeys which were infected by mosquitoes in the laboratory; microfilariae were found in six of them, and the seventh contained fertile female worms but died before microfilariae had been seen in the blood. Of these seven animals, five were *C. a. johnstoni*, one was *C. a. sabaeus*, and one *Macaca mulatta*. The source of the infection of all seven monkeys was M. 185. This monkey was easy to handle and had numerous microfilariae, and was therefore used more often than the other monkeys which were available at the time when most of the transmission experiments were carried out. Since so many *Cercopithecus aethiops* monkeys failed to become infected, no conclusion can be drawn from the failure to infect the single *Cebus* and *Cercocebus* monkeys. The monkeys which did not become infected were inoculated as follows: monkey 108, *C. a. johnstoni*, was inoculated on three occasions and received a total of 110 larval worms; monkey 151, *C. a. sabaeus*, two inoculations, 120 larvae; monkey 174, *C. a. johnstoni*, five inoculations, 765 larvae; monkey 193, *C. a. sabaeus*, three inoculations, 940 larvae;

monkey 195, *C. a. johnstoni*, five inoculations, 315 larvae; monkey 146, *Cebus apella*, two inoculations, 150 larvae; monkey 196, *Cercocebus* sp., three inoculations, 1310 larvae.

In a series of experiments more recent than those cited above, three monkeys were inoculated with larvae, and mosquitoes carrying infective larvae were allowed to feed on a fourth. The number of larvae used was smaller than in the earlier experiments and microfilariae were not found in the blood of any of these four monkeys up to 12 months after the last inoculation. Two of these monkeys had been inoculated unsuccessfully on previous occasions, and two had been infected but had lost their infections; consequently they may have possessed some immunity. Details were as follows: monkey 151, *C. a. sabaeus*, mosquitoes fed on the monkey on seven occasions, and it was estimated by sample dissections of mosquitoes before and after feeding that 50 larvae had been inoculated; monkey 174, *C. a. johnstoni*, four inoculations, 169 larvae; monkey 191, *C. a. johnstoni*, eight inoculations, 202 larvae; monkey 195, *C. a. johnstoni*, five inoculations, 148 larvae.

Prepatent period in monkeys infected in the laboratory

Monkeys 187 and 188 had been inoculated with infective larvae 42 and 47 weeks before microfilariae were first seen in the blood; monkey 189 had been inoculated 11, 41 and 47 weeks previously (Table 1). These three monkeys received worms from the same batch of mosquitoes on the same day and were found to have microfilariae in the blood at the same time as each other. This one batch of mosquitoes was probably therefore responsible for their initial infections, and the prepatent period was probably 47 weeks. Monkey 197 was inoculated 50 and 51 weeks before showing microfilariae in the blood. Monkey 194 was inoculated 22, 47 and 52 weeks before showing microfilariae; the batch used at 52 weeks failed to infect two other monkeys. The evidence from these five monkeys suggests that a period of 47–51 weeks is required before microfilariae appear in the blood. An apparent exception was monkey 164, which had been inoculated only 11 and 21 weeks before showing microfilariae; but this monkey had previously had a natural infection, so that these microfilariae could have been produced by the original worms, or by re-fertilization of one of the original females by a new male worm. At post-mortem examination of monkey 175, female worms containing living microfilariae were found, but no microfilariae had been detected in the blood. This monkey had been inoculated 12, 19, 39 and 64 weeks previously, but it is not clear which inoculation produced the infection.

Patent period

Table 2 shows the duration of microfilaraemia in monkeys which were infected in the laboratory and in monkeys which were infected naturally. In the monkeys infected in the laboratory microfilariae were first seen in the blood 11 months after the inoculation of infective larvae, but in the naturally infected monkeys the date when microfilariae first appeared in the blood was not known.

In five of the monkeys infected in the laboratory the number of microfilariae

in the blood at night increased to a maximum in 8–12 months. These monkeys were all *C. aethiops*, but in the rhesus monkey the maximum was found only 4 months after microfilariae first appeared in the blood. In one of the naturally infected monkeys the maximum was found 13 months after the first examination, but in the other four monkeys the interval was 1–5 months. The number of microfilariae therefore seems to increase for a period of about 1 year from the time that they are first present in the blood. The maximum number was 200–400 microfilariae per 20 mm.^{3} of blood in four of the monkeys infected in the laboratory, and 90 and 2000 per 20 mm.^{3} in the other two respectively.

Table 2. *Duration of microfilaraemia due to* Dirofilaria aethiops

Monkey	Maximum number of microfilariae per 20 mm.^{3} blood	Time of maximum microfilaria count (months after patency)	Time when microfilaria count fell to one-tenth maximum (months after patency)	Duration of patent period in months	Number of worms recovered at post-mortem	
					females	males
Laboratory infections						
164. *Cercopithecus aethiops johnstoni*	350	12	—	13	6	3
187. *C. a. johnstoni*	2000	12	—	13	25	8
188. *C. a. johnstoni*	370	9	24	27	5	10
189. *C. a. johnstoni*	200	12	21	34+	—	—
194. *C. a. sabaeus*	90	8	21	26	—	—
197. *Macaca mulatta*	200	4	7	20	10	3
175. *C. a. johnstoni*	—	—	—	—	3	—
Natural infections						
151. *C. a. sabaeus*	107	1	8	10	—	—
185. *C. a. johnstoni*	1238	5	7	24	2	4
190. *C. a. johnstoni*	37	3	10	10	1	3
191. *C. a. johnstoni*	150	2	9	21	—	—
192. *C. a. johnstoni*	172	15	24	24	3	1
Transplanted infection						
213. *C. a. johnstoni*	182	3	6	20	—	—

Of the six monkeys which had been infected in the laboratory, four had a greatly reduced microfilaraemia within 2 years of the date when microfilariae had first been found; the other two monkeys were killed shortly after the maximum number had been found. In most of the monkeys the total length of time during which microfilariae could be found in the blood did not exceed 2¼ years; in monkey 189, however, there were 1 or 2 microfilariae in 20 mm.^{3} of blood after 3 years. The number of microfilariae had diminished to one-tenth of the maximum in all the monkeys which survived for 2 years after the first microfilariae had been recorded.

Transplantation of adult worms

Adult worms were removed from three infected monkeys post-mortem and implanted into normal monkeys. Details were as follows:

Monkey 232. Three female and ten male worms from monkey 188 were implanted into subcutaneous pockets on the inner sides of the thighs of monkey 232, *C. a. johnstoni*. No microfilariae were found in the blood of this monkey.

Monkey 213. Two female worms and one male from monkey 164 were implanted into monkey 213, *C. a. johnstoni*. The muscles on the back and outer sides of the right thigh were split longitudinally in a fascial plane and the worms inserted between the muscle bundles. This took place 16 hr. after the death of monkey 164, but the worms were moving actively and appeared normal. Seven weeks later eight female and six male worms from monkey 187 were implanted into the same monkey. Three female and three male worms were inserted subcutaneously and intermuscularly into the back of the left thigh, and the remaining worms subcutaneously into the right groin and femoral triangle. Microfilariae were found in the blood of monkey 213, 3 months after the implantation of the worms from monkey 164; this was 2 weeks after the implantation of the worms from monkey 187. The maximum number of microfilariae was 180 per 20 mm.^{3} and was recorded 2 months after the microfilariae had first been found. After 6 months there were 13 microfilariae per 20 mm.^{3}, and up to 2 years after the first implantation of worms 1–2 microfilariae per 20 mm.^{3} were found. The patent period in this monkey was therefore at least 21 months. The adult worms had previously been responsible for microfilaraemia lasting 13 months in the original host monkey (whether this was monkey 164 or monkey 187), so the total duration of microfilaraemia due to these worms was nearly 3 years, a longer period than was found in any other monkey except monkey 189.

Location of the adult worms in the monkey

Five monkeys, 164, 187, 188, 197 and 175, which had been infected in the laboratory, and three naturally infected monkeys, 185, 190 and 192, have been examined for adult worms. The worms were found coiled in the superficial and

Table 3. *Distribution of adult* Dirofilaria aethiops *in host*

Monkey ...	164		175		187		188		190		192		197		Total	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Thigh	5	2	3	—	19	7	5	9	2	2	3	1	9	3	46	24
Leg	1	—	—	—	2	1	—	1	—	—	2	—	1	—	6	2
Trunk and upper extremity	—	—	—	—	3	—	—	—	—	—	—	—	—	—	3	—
Total	6	2	3	0	24	8	5	10	2	2	5	1	10	3	55	26

deep fasciae, on or between the muscle bundles; the majority were in the thighs, a few in the legs and a very few in other parts of the body. The distribution of the worms is shown in Table 3. Of the total of eighty-one worms, seventy were found in the thighs, eight in the legs, and in the most heavily infected monkey, three in

other parts of the body. No dead or encapsulated worms were found. The table also shows the marked preponderance of females over males. In *Litomosoides carinii* the ratio of females to males is approximately equal, but in *Dirofilaria aethiops* it is 2:1 among the worms recovered so far.

In histological sections the worms were found lying singly or in small groups in the fascial tissue on the surface of the muscle bundles. The space in which the worms lay was lined by a membrane, which in some places seemed like an endothelioid lining but was probably merely the result of compression of the connective tissue. Cells and microfilariae were both conspicuously absent from the space. There was no inflammatory reaction immediately round the worms but the adjacent connective tissue and its extensions between the muscle bundles showed evidence of chronic inflammation which varied in extent. The muscular tissue itself was not involved. In many parts the cells scattered through the connective tissue were increased in numbers—mostly fibroblasts and monocytes, but also a few lymphocytes and rare polymorphs—and there were often groups of cells around the small vessels. Lying in the connective tissue at some distance from the worms there were also dense collections of cells (lymphocytes, plasma cells, monocytes and fibroblasts, with few polymorphs); in the centre of these collections, many cells were degenerate with pycnosis and fragmentation of nuclei, but there was no sign of microfilariae in them. Microfilariae were remarkably scarce, but in one section a few microfilariae and partially developed ova were seen lying in the connective tissues at some distance from the worms and surrounded by cellular reaction.

DISCUSSION

This work was undertaken in the hope of adding to the small number of filariae which are available for laboratory investigations. The infection has already been used to study periodicity (Hawking & Thurston, 1951; McFadzean, 1952), for which it was particularly valuable because the periodicity of the microfilariae is more marked than in *Dirofilaria* in dogs and resembles the cycle in *Wuchereria bancrofti* in man.

Transmission

Unfortunately the infection has not been fully established, although it has been shown that it would be possible to do so. The parasite was transmitted to seven normal monkeys from a green monkey which came from Africa with a naturally acquired infection. In this first series of transmissions it was apparent that *Aëdes aegypti* was not a very efficient vector and that the majority of the larvae which were inoculated into the monkeys failed to become adult worms. For various reasons few mosquitoes were available to us at the time when the infection in the seven monkeys was most favourable for further transmission, and many of the mosquitoes used in the second series of inoculations were infected by monkeys whose microfilaraemia was declining. In addition, since the supply of green monkeys was limited, animals were used as recipients which had resisted or overcome previous infections and may therefore have had some degree of immunity. The combination of all these factors probably accounts for the failure of the later series of inoculations.

Host specificity

Filarial infections are usually specific to the vertebrate host, although there are several recorded cases of filariae of dogs being found in man. Accordingly, it was surprising that a filaria from a green monkey developed so well in monkey 197, a rhesus monkey. The only indication of unsuitability of the host was that the maximum number of microfilariae was present 4 months after they had first been found, and the number fell to one-tenth of the maximum in 7 months, whereas in the green monkeys the corresponding periods were 8–12 and 21–24 months. At the post-mortem examination the female worms were found to contain microfilariae which appeared normal, but immediately before death very few had been present in the peripheral circulation. The reduction in the numbers of circulating microfilariae may have resulted from increasing immunity of the host so that either the microfilariae themselves were destroyed, and/or the fertility of the female worms was reduced. Since only a single *Cercocebus* monkey and a single *Cebus apella* were inoculated, no conclusion can be drawn concerning the susceptibility of these species to the worm. The two species of mosquito which were investigated as intermediate hosts were both found to allow development of microfilariae to infective third-stage larvae. The fact that *Aëdes aegypti* was more favourable to the worms than *Anopheles maculipennis atroparvus* does not necessarily mean that the natural vector is more likely to be a Culicine than an Anopheline mosquito.

Adult worms

From the histological examination of the worms in section in the tissues it is not clear whether the worms migrate frequently through the tissues, as *Loa loa* does, or, like *Onchocerca volvulus*, stay permanently in one position and become encapsulated. Since the tissue reaction round the worms is limited, it seems probable that *Dirofilaria aethiops* is much better adapted to its host than *Onchocerca* and/or that it does not remain in one situation for such long periods.

Apart from slight chronic inflammation near the worms, no pathogenic effects have been observed. The number of animals studied, however, is too small to be certain that harm is not occasionally produced by the infection.

In *Wuchereria bancrofti* microfilariae are found in the lymph near the adult worms (Lane, 1948), and in *Onchocerca volvulus* there are microfilariae in the nodules containing the worms. In *Dirofilaria aethiops*, however, no microfilariae were found in the space round the worms and very few in the adjacent tissues. In six preserved female worms from one monkey, microfilariae were found in the uteri only 2·9–5·9 mm. behind the vulva and the blood contained many microfilariae up to the day before the monkey died. The microfilariae probably therefore pass quite rapidly from the space round the worm into the blood stream. Alternatively, the microfilariae may be discharged at infrequent intervals, so that there are long periods in which no microfilariae remain near the parent worm.

Comparison of Dirofilaria aethiops *with* Dirofilaria repens

D. aethiops is similar in structure to *D. repens*, a filarial parasite of dogs, and we have found that the two species also resemble each other in habitat and life history. Studies of *D. repens*, which were carried out in this laboratory, have been described elsewhere (Hawking & Thurston, 1951; Mellanby, 1956; Webber & Hawking, 1955). The adult worms of both species are parasites of the connective tissue of their mammalian hosts and the microfilariae live in the blood. The microfilariae are periodic with the maximum number in the peripheral circulation at midnight and the minimum at midday. The larvae develop to the infective third stage in several species of mosquitoes and the infective larvae are inoculated into the definitive host when the mosquitoes feed.

Detailed study of the life history reveals differences between the species. The periodicity of the microfilaria is more marked in *D. aethiops* than in *D. repens*, the number in the peripheral circulation in the day-time being 1 % of the number at night in *D. aethiops* and 25 % in *D. repens*. *D. aethiops* develops more slowly than *D. repens* in the mosquito and has a longer pre-patent period in the vertebrate, but the duration of the rise in the microfilaria count and of the total patent period are shorter. Although both species live in the connective tissue of the mammalian host, *D. aethiops* occurs in the connective tissue of the muscle bundles, and *D. repens* in the connective tissue under the skin. *D. aethiops* occurs most frequently in the thighs and *D. repens* on the trunk, but the distinction is not rigid because individuals of both species can occasionally be found in almost any region of the trunk and limbs. In the intermediate host the tissue preference is more marked; *D. aethiops* develops in the subcuticular connective tissue but *D. repens* develops in the Malpighian tubules. Three species of mosquitoes have been investigated as intermediate hosts; *D. aethiops* developed best in *Aëdes aegypti*, not very well in *Anopheles maculipennis atroparvus* and not at all in *A. stephensi*; *Dirofilaria repens* developed best in *Anopheles stephensi* and moderately well in *A. m. atroparvus* and *Aëdes aegypti*. The number of larvae which survived and the time required for development to the infective third stage was different for each species of filaria and mosquito.

SUMMARY

1. An account is given of the stages of the life history of *Dirofilaria aethiops* which take place in the monkey and of the transmission of the infection in the laboratory.

2. The microfilariae exhibit nocturnal periodicity in the blood; the number of microfilariae at midday was usually less than 1 % of the number at midnight.

3. *Aëdes aegypti* mosquitoes were fed on monkeys during the evening when there were many microfilariae in the blood. Larvae developed in these mosquitoes and they were ultimately transferred to fresh monkeys by injection of a saline suspension of mosquitoes and infective larvae. In this way six *Cercopithecus aethiops* and one *Macaca mulatta* monkey were successfully infected; seven other *Cercopithecus aethiops*, one *Cebus apella*, and one *Cercocebus* monkey were inoculated, but the worms failed to establish themselves.

4. In the infected monkeys microfilariae appeared in the blood 11 months after the injection of infective larvae. The number of microfilariae then increased for 8–12 months. Subsequently, it decreased to one-tenth of the maximum in 2 years from the first appearance.

5. Post-mortem examination showed that the adult worms live in the fascial tissues on or between the muscle bundles, especially those of the thighs. Tissue reaction to the worms is slight and in these monkeys there was no other pathogenic effect of the infection.

6. Adult worms from three of the monkeys were transplanted to two normal monkeys. Microfilariae were found in the blood of one of these and persisted for over 2 years.

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DIROFILARIA AETHIOPS WEBBER, 1955, A FILARIAL PARASITE OF MONKEYS

III. THE LARVAL DEVELOPMENT IN MOSQUITOES

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(With 16 Figures in the Text)

In the previous papers of this series (Webber, 1955*b*; Hawking & Webber, 1955) descriptions were given of the stages of *Dirofilaria aethiops* (Webber, 1955*a*) which have been found in monkeys (*Cercopithecus aethiops*) and of the transmission of this filaria in the laboratory. In the present paper the stages which take place in the intermediate host are described. It was found that *Dirofilaria aethiops* could develop in both *Aëdes aegypti* and *Anopheles maculipennis atroparvus*, but the former species appeared to be the more favourable to the larvae. The morphology, migration and survival of the larval stages will therefore be described as found in *Aëdes aegypti*.

The mosquitoes were infected by allowing them to feed on an infected monkey. At a time in the evening when there were 200–400 microfilariae in 20 mm.^{3} of blood, the monkey was anaesthetized with parenteral barbitone and placed in a cage of mosquitoes for 20–30 min. The mosquitoes were dissected at intervals after their feed on the infected monkey to trace the development of the filarial larvae. Some hundreds of mosquitoes were dissected in the course of about 2 years. At 26° C. and 80% relative humidity development to the infective third stage larva was completed in 18–20 days.

THE MORPHOLOGY OF THE LARVAL STAGES IN *AËDES AEGYPTI*

(1) *First-stage larva*

First and second days (Figs. 1, 2). The microfilaria or first-stage larva as it occurs in the blood and in the stomach of the mosquito is similar in organization to microfilariae of many other species. It is unsheathed, and the tail tapers to a filament which does not contain nuclei. Two types of nucleus are present in the nuclear column and they are most easily distinguished by using vital stains of the methylene-blue group. The first type of nucleus is elongated and stains rapidly with Azur II; the nuclei of this type are found in two superficial rows along the body of the microfilaria and they correspond to the muscle cells of Rodenwaldt (1933). These nuclei can be seen throughout the first stage of larval development, and they are shown in Fig. 3 in a larva which had developed in 4 days in a mosquito. The remaining nuclei are more spherical and take up vital stains of the methylene-blue group more slowly. In addition to these nuclei the nucleus of the excretory cell is sometimes seen in vital-stained preparations as a pale nucleus with a dark nucleolus; this contrasts with the other nuclei which stain uniformly dark and therefore do not show a nucleolus. At this stage (1 day) the rectal cells cannot be

demonstrated. In fixed and dried preparations the cytoplasm is eosinophilic and the nuclei strongly basophilic.

After 2 days in the mosquito, the excretory cell is easily seen in most larvae about 10μ behind the excretory pore. Large rectal cells which stain less intensely than the nuclei of the nuclear column are also present in many larvae. The excretory and rectal cells can now be seen both in vital-stained preparations and in sections of mosquitoes stained with haematoxylin and eosin (Fig. 2).

Third and fourth days (Fig. 3). After the second day the staining properties of the nuclei and of the cytoplasm begin to change. The cytoplasm becomes weakly basophilic so that the contrast between nuclei and cytoplasm weakens. By the fourth day the nuclei of the nuclear column are of two distinct types: those which still stain deeply and those in which a dark nucleolus is surrounded by a paler region. The total number of nuclei and the proportion of nuclei of the second type increases, and the excretory and rectal cells enlarge until they occupy almost the whole width of the larva. At the same time the width of the larva increases but the length decreases slightly. The nuclei in the anterior third of the larva now begin to be more closely packed; but those in the middle third are more scattered (Fig. 3).

Fifth to seventh days (Figs. 4, 5). The first organ to appear is the gut (Fig. 4, *int.n.*), consisting of three independent parts—rectum, intestine and oesophagus. The rectum is a spheroidal structure, connected to the surface of the body by a short wide tube which reaches to the anal pore. The anal pore of the microfilaria thus becomes the anus of the developing worm. The rectum becomes lined with cuticle which projects in a shallow dome from the anus (Fig. 5, *r.pl.*). The intestine is formed in the mid-region of the body, and the oesophagus in the anterior region. Both at first are solid columns of cells; later they become hollow, although the oesophageal and intestinal lumina do not communicate with each other. The oesophageal lumen becomes lined with cuticle which is continued forwards into the stoma, but the latter is not yet open to the exterior. At this stage the nerve ring consists of a number of small cells, but no fibres are visible. The excretory cell remains large.

Eighth to twelfth days (Fig. 6). Shortly before the first moult the nuclei of the gut divide rapidly; by the tenth day the lumina of the oesophagus and intestine are continuous. The intestine sometimes contains small refractile droplets (Fig. 6), but the lumen of the intestine does not open into the rectum. The excretory cell comes to lie closer to the excretory pore until they are in contact. On each side of the larva there is a row of elongated hypodermal nuclei which correspond in position to the darkly staining 'muscle cells' of the first-stage larva (see above) and to the lateral cords of the infective larva and adult worm. It seems, therefore, that the hypodermal nuclei can be traced through all the stages from the microfilaria to the adult worm. In the first-stage larva the tail is practically unchanged and its nuclei do not take part in the changes undergone by the rest of the body.

The first moult occurs at about the 12th day. The external cuticle and the linings of the stoma, oesophagus and rectum are shed. The last four nuclei of the tail come away inside the shed cuticle (Fig. 7, *l.n.* I).

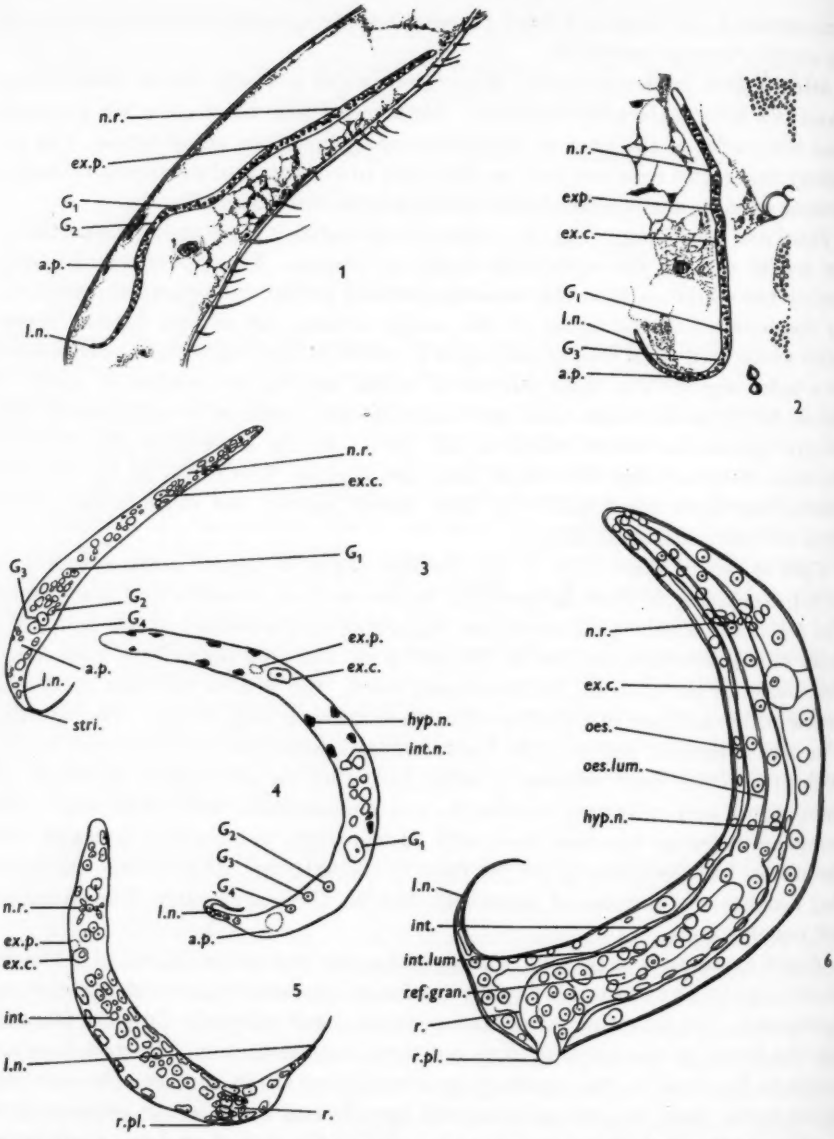


Fig. 1. Section of labium of *A. aegypti* containing first-stage larva of *D. aethiops*, 1 day after the mosquito had been infected. Drawn from serial sections stained with haematoxylin and eosin. × 400.

Fig. 2. Section of thorax of *A. aegypti* containing first-stage larva of *D. aethiops*, 2 days after the mosquito had been infected. Drawn from serial sections stained with haematoxylin and eosin. × 400.

Fig. 3. *D. aethiops*, first-stage larva, after 4 days in *A. aegypti*. × 400.

Fig. 4. *D. aethiops*, first-stage larva, after 5 days in *A. aegypti*. Stained with Azur II to show hypodermal nuclei. × 400.

Fig. 5. *D. aethiops*, first-stage larva, after 7 days in *A. aegypti*. × 400.

Fig. 6. *D. aethiops*, first-stage larva, after 9 days in *A. aegypti*. × 400.

(2) *Second-stage larva*

Twelfth to sixteenth days (Fig. 7). At the time of the first moult the principal organs of the larva have already been formed. Subsequent development consists of elaboration of the organ system and of changes in the relative lengths of the regions of the body. In the second-stage larva the stoma opens to the exterior and there are nearly always refractile granules round the base of the mouth. In the

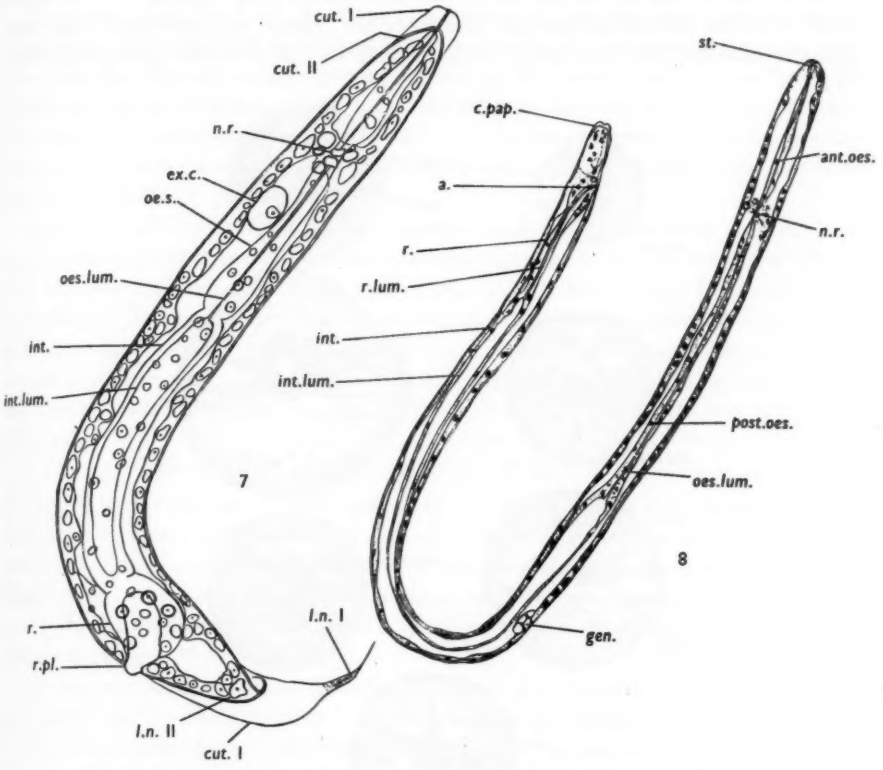


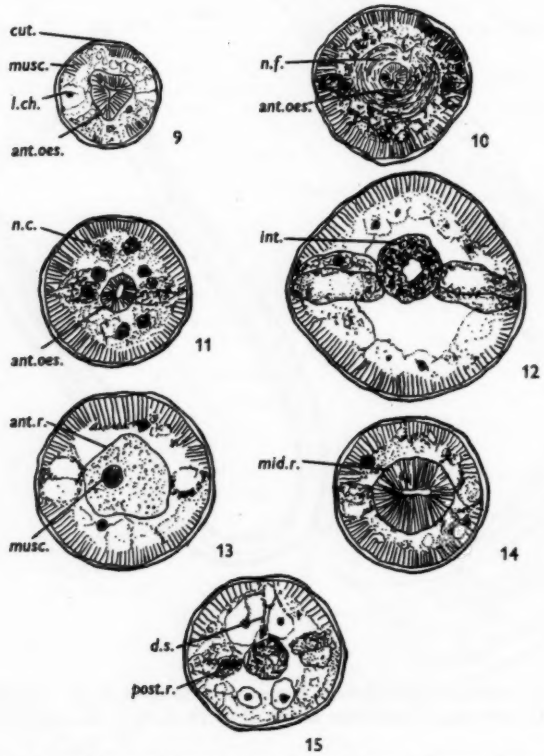
Fig. 7. *D. aethiops*, second-stage larva, after 15 days in *A. aegypti*. × 400.
Fig. 8. *D. aethiops*, third-stage larva, after 18 days in *A. aegypti*. × 200.

nerve ring fibres are visible and the cells are more compactly arranged than before. The excretory cell remains large. The lumen of the intestine is much larger, compared with the thickness of the wall of the intestine, than in the previous stage. The space between the gut and the body wall is much more clearly defined, especially in the rectal region. A sheet of cells stretches from the rectum to the dorsal body wall. In one specimen a single large cell was seen just behind the junction of oesophagus and intestine; presumably it was the first cell of the genital system. The cuticle at the caudal end is similar in shape to the tail of the first-stage larva, but the protoplasm ends in a small knob at the point where the tail

narrows to a curved cuticular filament. During the second stage the larva grows in length principally in the intestinal region, and moves more and more actively. The second moult occurs at about the sixteenth day.

(3) *Third-stage larva*

In the third stage the stoma is a short cuticular tube leading to the triradiate lumen of the oesophagus. The gut is now open from mouth to anus, as the intestine communicates with the rectum and there is no rectal plug. The larvae will ingest solid particles, such as indian ink, and there are regular contractions of the oesophageal muscles. The muscles of the body wall are visible in the intercordal areas



Figs. 9–15. *D. aethiops*, sections of third-stage larva, drawn from sections of the proboscis of *A. aegypti*. × 1600.

Fig. 9. Anterior muscular part of the oesophagus.
Fig. 10. Anterior muscular part of the oesophagus, at the level of the nerve ring, showing nerve fibres.
Fig. 11. Anterior muscular part of the oesophagus, at the level of the nerve ring, showing nerve cells.
Fig. 12. Intestinal region.
Fig. 13. Anterior glandular part of rectum.
Fig. 14. Muscular part of rectum.
Fig. 15. Dorsal septum and posterior part of rectum.

as longitudinal striations beneath the cuticle. The nerve ring possesses fibres and cells (Figs. 10, 11); and cells similar in form to the nerve cells are scattered as far back as the end of the oesophagus. The oesophagus is divided into anterior and posterior regions (Fig. 8, *ant.oes.*, *post.oes.*): in front of the nerve ring its wall is thinner; behind the nerve ring it is thicker and the cell cytoplasm is more granular. The so-called genital anlage can be seen in about one-third of the worms as a compact group of six to eight cells, which lie 10μ behind the oesophagus (Fig. 8, *gen.*). The tail is blunt and carries three terminal papillae (Fig. 8, *c.pap.*).

Diagrams of sections of the third-stage larva in the proboscis are shown in Figs. 9–15. There is a well-developed cuticle and a thin hypodermal layer with large lateral cords. The muscular layer is divided by the lateral cords, there being no dorsal or ventral division; the dorsal musculature and the ventral musculature therefore each contract and relax as one unit. In the most anterior and posterior parts of the worm, however, the muscle layer is thinner and probably divided into four bands (Figs. 9, 15).

Anteriorly the oesophagus has a muscular wall and the lumen is triradiate in cross-section; further back the lumen is circular in cross-section and more posteriorly still the wall is glandular. The intestine (Fig. 12, *int.*) has a larger lumen than the oesophagus. In the anterior part of the rectum a narrow layer of muscle surrounds the rectal lumen, but most of the thickness of the wall is glandular tissue (Fig. 13). In the posterior part of the rectum the wall is entirely muscular (Fig. 14), and in the cloacal region a dorsal septum connects the rectal wall with the body wall (Fig. 15).

MIGRATION OF THE LARVAE IN THE MOSQUITO

In recording the degree of development of the larvae it was convenient to define four forms. These four forms did not correspond with the stages delimited by moults, but they were easily recognizable under a low power of the microscope and they enabled the migration of the larvae to be followed conveniently.

(1) *Young larvae:* 200 × 4μ to 250 × 20μ. This term was used for any larva before the sausage form.

(2) *Sausage forms:* 250–400μ in length, 25–35μ in breadth. The ratio of length to breadth fell to about 10 and the larvae were very sluggish.

(3) *Post-sausage forms:* 400–600μ in length, 25μ or more in breadth. This term was used for forms which were longer than the sausage form and more active, but which were not yet infective larvae.

(4) *Infective larvae:* more than 600μ in length, 20–25μ in breadth. Some larvae reached 1000μ but the majority were 700–800μ long by the time they reached the proboscis.

The larvae of *Dirofilaria aethiops* develop in the connective tissue. Consequently they are not confined to the thorax or abdomen but can be found in any part of the mosquito body where there is connective tissue: thus young larvae may occur in the labium and infective larvae may occur in the abdomen. This is in contrast with the larvae of *Wuchereria* which develop between the thoracic muscles, and the larvae of *Dirofilaria repens* and *D. immitis* which develop in the Malpighian tubules.

Table 1 is based on dissections of mosquitoes of many batches; it shows the distribution of the four stages of development between the proboscis, head, thorax and abdomen of the mosquito from the first to the twenty-fourth day after the infecting feed. Throughout the 24 days more larvae were found in the abdomen than in any other region of the body. The larvae began to leave the stomach after 24 hr. and most of them had escaped within 3 days. On the second day the great majority were still in the abdomen, but by the third to fifth days some were in the thorax and head. On the fifth day nearly 60 % were in the abdomen, 40 % in the thorax and a few (about 3 %) in the head.

From the fifth to the eleventh day, while they were sausage forms, the larvae were much less active than before. The distribution of larvae between the different body regions of the mosquito therefore changed very little. By the end of this period, however, the larvae were again becoming active and there were more post-sausage and infective forms than sausage forms and young larvae.

From the twelfth day onwards the larvae became more and more active. They moved to the anterior part of the mosquito, and infective larvae reached the proboscis. There were, however, still many sausage forms in the rest of the mosquito, particularly in the abdomen where they persisted in diminishing numbers at least to the twenty-fourth day.

Thus, development shows three phases: in the first phase the active young larvae leave the stomach and migrate to the connective tissue; in the second phase the larvae are inactive and undergo structural re-organization; in the third phase activity is resumed, and the larvae move forward to the proboscis from which they eventually escape.

SURVIVAL OF THE LARVAE IN THE MOSQUITO

The number of microfilariae which the mosquito obtains when it feeds on an infected monkey depends on the size of the blood meal and the density of the microfilariae in the blood. The relation between these factors was investigated by recording the weight of blood taken by individual mosquitoes, the number of microfilariae they obtained, and the number of microfilariae in the blood of the monkey.

The mosquitoes were lightly anaesthetized with ether, weighed, and confined in half test-tubes. The tubes were inverted one at a time over the ear or chin of an infected monkey. During, or shortly after feeding, blood samples were taken from the ear of the monkey. It was hoped that the mosquitoes would feed on the ear, but only two did so and the remaining twenty-one fed on the chin. Immediately after they had fed, they were re-weighed and then teased out on a slide. The blood films from the monkey and the teased preparations of the mosquitoes were later stained and examined for filarial larvae. The increase in weight after feeding was usually 3–5 mg., although one individual increased by 5·6 mg. and six mosquitoes took only partial meals of 1·2–2·9 mg. The number of larvae ingested was 2–30, which was a greater proportionate range than the range in the number of microfilariae in the blood of the monkey. The average number of larvae ingested was less than the number which would have been expected on the basis of the number in the blood of the monkey. The difference was not great, however, especially in

Table 1. *Development of* Dirofilaria aethiops *in* Aëdes aegypti

ays fter fec- on	Abdomen				Thorax				Head				Proboscis I	Abdomen	Thorax	Head	Proboscis	Abdomen	Thorax	Head	Proboscis
	Y	S	PS	I	Y	S	PS	I	Y	S	PS	I									
	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)
1	12	—	—	—	—	—	—	—	—	—	—	—	—	539	1	—	—	551	1	0	0
																		99·8	0·2	0	0
2	20	—	—	—	20	—	—	—	—	—	—	—	—	14	1	—	—	34	21	0	0
																		62	38	0	0
3	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	—	3	0	0	0
4	—	—	—	—	—	—	—	—	—	—	—	—	—	49	10	2	—	49	10	2	0
																		80	16	3	0
5	25	11	—	—	1	6	—	—	—	—	—	—	—	25	36	3	—	61	43	3	0
																		57	40	3	0
6	—	—	—	—	—	—	—	—	—	—	—	—	—	12	8	4	—	12	8	4	0
																		50	33	17	0
7	2	—	—	—	—	—	—	—	—	—	—	—	—	12	17	2	—	14	17	2	0
																		42	52	6	0
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	1	11	—	—	3	6	—	—	6	4	—	—	—	2	12	1	—	14	21	11	0
																		31	45	24	0
0	21	22	—	—	2	10	—	—	—	—	—	—	—	14	8	—	—	46	19	0	0
																		71	29	0	0
1	1	4	1	4	1	2	—	4	—	—	1	—	—	—	—	—	—	10	7	1	0
																		56	39	5	0
2	—	11	2	3	—	3	6	1	—	1	4	1	2	—	—	—	—	16	10	6	2
																		47	29	18	6
3	6	6	3	—	2	11	2	—	—	—	—	—	—	26	16	—	—	41	31	0	0
																		57	43	0	0
4	—	23	11	1	—	6	6	1	—	—	1	—	—	—	—	—	—	35	13	1	0
																		72	26	2	0
5	—	8	12	13	3	3	15	11	—	—	1	5	3	6	3	4	—	39	35	10	3
																		45	40	12	3
6	—	4	10	5	—	2	3	3	—	—	1	—	—	4	3	—	1	23	11	1	1
																		64	30	3	3
7	—	—	—	—	—	—	—	—	—	—	—	—	—	6	1	2	1	6	1	2	1
																		60	10	20	10
8	—	13	2	12	—	6	3	—	—	—	1	4	2	—	—	—	—	27	9	5	2
																		62	21	12	5
9	—	2	3	14	—	3	—	7	—	1	—	5	5	—	—	—	—	19	10	6	5
																		47	25	16	12
0	—	2	7	12	—	1	2	6	—	—	1	6	5	—	—	—	—	21	9	7	5
																		50	21	17	12
1	—	6	2	14	—	—	2	5	—	—	—	3	1	—	—	—	—	22	7	3	1
																		67	21	9	3
2	—	1	5	12	—	—	—	1	—	—	—	4	6	—	—	—	—	18	1	4	6
																		62	3	14	21

Y = young larvae; S = sausage stage; PS = post-sausage stage; I = infective larvae.

Columns 2–18: figure = no. of larvae; columns 19–22: upper figure = no. of larvae, lower figure = % of rvae; columns 15–18 give results of dissections where larval stage was not recorded; columns 19–22: give als for body regions.

comparison with the variation from one mosquito to another. The actual number of microfilariae obtained by a mosquito was greater when a large meal was taken and when there were more microfilariae in the blood of the monkey (Table 2).

Table 2. *Number of microfilariae of* Dirofilaria aethiops *ingested by* Aëdes aegypti *feeding on* Cercopithecus aethiops

No. of mosquitoes	Volume of blood ingested (mm.³)	No. of microfilariae per mosquito	Number of microfilariae per 20 mm.³ blood: Calculated per mosquito	Number of microfilariae per 20 mm.³ blood: Found in monkey
4	4·6 ± 1·0	8 ± 4	36 ± 14	56 ± 6
5	3·9 ± 0·5	10 ± 5	55 ± 26	63 ± 4
6	3·9 ± 0·4	14 ± 6	74 ± 22	86 ± 15
7	2·4 ± 0·5	7 ± 3	64 ± 17	88 ± 14

The above observations were made when the infection in the monkey was waning and the number of microfilariae in the blood was less than 100 per 20 mm.³ at 7.30 p.m. The average number of microfilariae obtained by each mosquito was approximately one-sixth of the number found in 20 mm.³ of ear-blood. When the infection in the monkey was rising or was still at the maximum, and there were 200–400 microfilariae per 20 mm.³ of blood in the early evening, it was estimated, by examining fresh teased preparations, that the mosquitoes obtained 5–100 microfilariae, or about one-quarter of the number found in 20 mm.³ of blood. As in the mosquitoes fed on the monkey with the waning infection, the number of microfilariae showed great variation from one mosquito to another; the highest number found was 1000. Thus the number of microfilariae ingested by mosquitoes was found to be variable, and this perhaps depends on an uneven distribution of microfilariae in the blood vessels. There was no evidence that the mosquitoes concentrated the microfilariae.

On the morning following a feed on a monkey with a rising or maximal infection, the average number of larvae found per mosquito was sixty (Fig. 16). On the second day after the feed the average number had fallen to five. Survival after the second day, i.e. survival of those larvae which had penetrated the gut wall successfully, was much better, so that at 20 days there was an average of one larva per mosquito. Table 1 shows that the rate of development of the larvae was very variable. Although the time for the most advanced larvae to reach the infective stage in the proboscis was constant under constant environmental conditions, there were always many larvae at earlier stages at this time.

In *Anopheles maculipennis* even fewer larvae than in *Aëdes aegypti* reached the infective stage. Young larvae were found as late as the eleventh day; there were a few larvae which were sausage forms in shape and size but which had no organization of the cells; and at 20 days the proportion of infective larvae to total larvae was even smaller than in *Aëdes*.

COMPARISON OF LARVAL STAGES OF *DIROFILARIA AETHIOPS* WITH THOSE OF OTHER FILARIAE

The development of *D. aethiops* in the mosquito may be compared with that of *Wuchereria malayi* which has been described in detail by Feng (1936). The morphological changes in *D. aethiops* resemble the changes seen in *W. malayi* closely and only minor differences can be found. At all stages the larva of *W. malayi* is larger

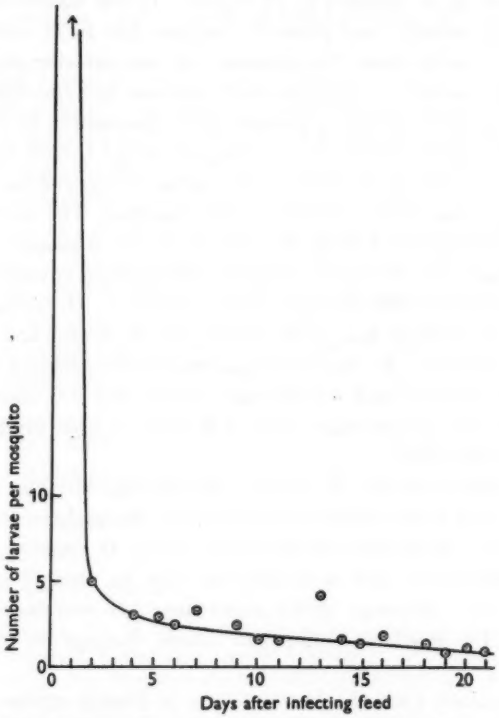


Fig. 16. Graph to illustrate the survival rate of *D. aethiops* larvae in *A. aegypti*.

than that of *D. aethiops*. The microfilariae of *W. malayi* are 200–250 μ, and those of *D. aethiops* are 200 μ; the infective larvae of *W. malayi* are 1300 μ while *D. aethiops* are 900 μ. Before the last nuclei of the tail are lost at the first moult, *W. malayi* shows two to three small nuclei followed by one larger nucleus while *D. aethiops* has three to four elongate nuclei. When they have left the stomach of the mosquito, the larvae of *W. malayi* live between the thoracic muscles; *D. aethiops* lives in the connective tissue. In *W. malayi* the infective third stage larva was reached in 6½ days, a very much shorter time than the 18–20 days required by *D. aethiops*. This difference was no doubt accentuated by environmental conditions. Feng kept *Anopheles hyrcanus* var. *sinensis* at 29–30° C., but in the present work *Aëdes aegypti* was kept at 26–27° C. In addition, *An. hyrcanus* is a natural vector of *W. malayi* but *Aëdes* is probably not the natural vector of *D. aethiops*. The head of the third-

stage larva of *W. malayi* carries eight papillae and two amphids, and there are also said to be lip-like structures. In *D. aethiops* the head papillae, although almost certainly present, are too small to be visible. The rest of the structure of the third-stage larvae, including the caudal papillae, is identical in the two species.

Although *D. repens* is approximately the same size as *D. aethiops*, it was found that the structures in *D. repens* were more clearly defined and delimited. The two parts of the oesophagus showed the same type of differences in the two species, but the differences were more marked in *D. repens*. In the latter there is a distinct groove between the anterior and posterior regions, but in *D. aethiops* the regions taper gradually into each other. The diameter of the anterior region is less than the diameter of the posterior region in both species, but the difference is greater in *D. repens*. The genital anlage is present more frequently in *D. repens* than in *D. aethiops*, and is easily visible at the two-cell stage before the second moult. Eight papillae can be seen on the head of *D. repens*. *D. repens* has one large caudal papilla, *D. aethiops* has three smaller caudal papillae. The larvae of *D. repens* cannot easily be distinguished from *D. aethiops* in the stomach; when they leave the stomach the fact that *D. repens* is in the Malpighian tubules and *D. aethiops* is in the connective tissue differentiates them. At 26° C. *D. repens* develops to the infective stage in 11–12 days in a good vector (*An. stephensi*) and in 14–16 days in a poor vector (*A. aegypti*). At the same temperature *D. aethiops* takes 18–20 days both in *A. aegypti*, which is not a very good vector and *An. maculipennis*, a poor vector. Thus these two species show some difference in host preference and in the time taken for development.

D. immitis closely resembles *D. repens*; morphologically the larval stages are indistinguishable, and both species develop in the Malpighian tubules and in the same length of time. According to Summers (1943) *D. immitis* develops best in *Ctenocephalides* and *Culex* but will also develop in several species of *Aëdes*, *Anopheles* and *Culex*, although *Aëdes infirmatus*, *A. sollicitans*, *A. guamensis*, *A. pandani* and *An. crucians* suffer too much damage to be very effective vectors.

It may be concluded that the larval forms of filarial worms are more easily differentiated by the characteristics of their life cycles than by their morphology. Each species of filaria can develop in several intermediate hosts, but in some host species development is complete, while in other host species it is incomplete. Conversely, most vector species support development of more than one filarial species. The species of filaria are characterized therefore by the range of species which can act as their intermediate hosts, and by the degree of development attained in these various vector species. The length of time required for development is a characteristic of the filarial species, but varies with the host and climatic conditions. Up to the end of the first stage, the larvae can be recognized by the structure of the tail, which is the same as in the microfilaria in the blood. The second-stage larvae are virtually indistinguishable on morphological grounds but have characteristic sites of development in the vector. The third-stage larvae show some variation in the cephalic and caudal papillae.

SUMMARY

1. A description is given of the morphological development of the larval stages of *Dirofilaria aethiops* in the mosquito *Aëdes aegypti*.

2. The first-stage larvae migrate from the stomach to the connective tissue in all parts of the mosquito body, where they pass through the inactive 'sausage' stages. At 26° C. and 80 % relative humidity the first moult takes place about the twelfth day of development and the second moult about the sixteenth day. The third-stage larvae migrate towards the proboscis where they can be found after 18–20 days.

3. The number of microfilariae ingested by the mosquito is approximately equal to the number which would be expected from the numbers in the blood of the monkey and the volume of the blood meal. The death rate of the larvae in the mosquitoes is very high for the first 2 days, but less severe later. Approximately 1 % of the ingested microfilariae survive 20 days; at this time two-thirds of the surviving larvae have reached the third stage.

4. Comparison of *D. aethiops* with other filariae shows that first-stage larvae of different species can often be distinguished by the structure of the tail, which is the same as that of the microfilaria. Second-stage larvae are similar to each other in morphology but develop in characteristic sites in the host. Third-stage larvae differ from each other in the cephalic and caudal papillae.

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List of abbreviations in figures

a.	anus	*l.n.* II	last nucleus of tail of second-stage larva
a.p.	anal pore	*m.*	mouth
ant.oes.	anterior muscular part of oesophagus	*mid.r.*	mid-region of rectum
ant.r.	anterior part of rectum	*musc.*	muscle
c.pap.	caudal papilla	*n.c.*	nerve cell
cut.	cuticle: *cut.* I, cuticle of first-stage; *cut.* II, cuticle of second-stage	*n.f.*	nerve fibre
d.s.	dorsal septum	*n.r.*	nerve ring
ex.c.	excretory cell	*oes.*	oesophagus
ex.p.	excretory pore	*oes.lum.*	lumen of oesophagus
G	'G' cells	*post.oes.*	posterior glandular region of oesophagus
gen.	genital anlage	*post.r.*	posterior part of rectum
hyp.n.	hypodermal nuclei	*r.*	rectum
int.	intestine	*r.lum.*	lumen of rectum
int.lum.	lumen of intestine	*r.pl.*	rectal plug
int.n.	nuclei of intestine	*ref.gran.*	refractile droplets in intestine
l.ch.	lateral cord	*st.*	stoma
l.n.	last nucleus of the tail: *l.n.* I, last nucleus of tail of first-stage larva;	*stri.*	cuticular striations

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THE FILARIAL WORMS *DIPETALONEMA DIGITATUM* AND *D. GRACILE* IN MONKEYS

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(With 7 Figures in the Text)

This paper describes a laboratory study of natural infections of *Dipetalonema digitatum* (Nematoda: Filariidae) in *Macaca speciosa* and *Dipetalonema gracile* in two *Cebus apella* monkeys.

Dipetalonema digitatum (Chandler, 1929)

Origin and general history

The macaque monkey (monkey 145) came to this country from India, where it was said to have been obtained near Chittagong, Bengal. Microfilariae were found in the blood at 9.30 a.m. during April 1948, when the monkey weighed 1·6 kg. and was said to be 9 months old. Blood films were taken at 9.30 a.m. from five other similar young monkeys, but no microfilariae were seen in them. The monkey was used for various experiments on the periodicity of microfilariae which have been reported elsewhere (Hawking & Thurston, 1951). It was killed on 16 July 1948. There was no evidence that the infection had had any harmful effect on the monkey.

Morphology of the adult worms (Figs. 1–3; Table 1)

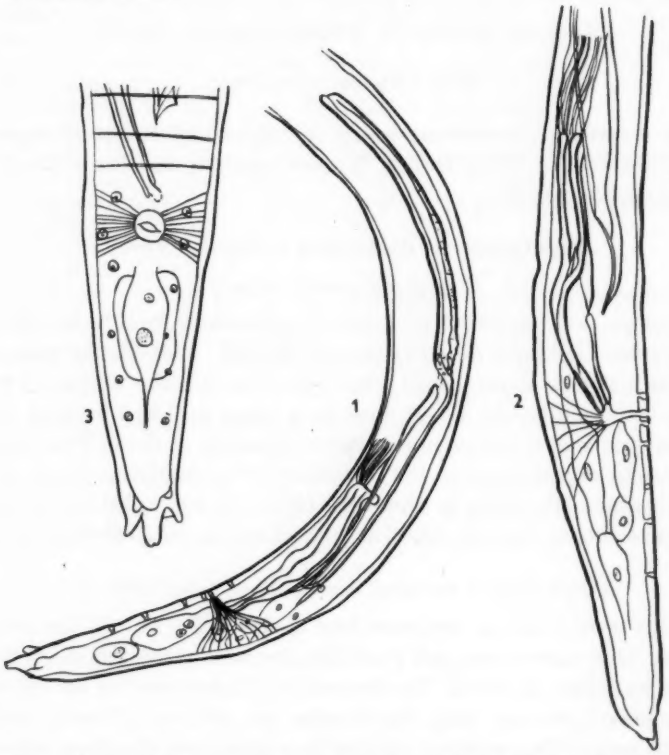
The worms were found in the peritoneal cavity, lying among the folds of the mesenteries. One male worm and three females were preserved in glycerine and alcohol and examined in detail. The dimensions of these worms are shown in the table. The male is 80 mm. long; the females 194, 205 and 216 mm. respectively (average 205 mm.). The cuticle is striated in a transverse direction, especially on the ventral surface of the tail of the male. The head is rounded, and four pairs of cephalic papillae are visible. The mouth opens without lips or other ornamentation into the oesophagus, which is 0·86 mm. long in the male and 1·0 mm. long in one of the females. The anterior part of one female is damaged. The transition from oesophagus to intestine is gradual, occurring in the female at the level of the vulva; it is therefore difficult to be certain of its exact position. The tail is 116μ long in the male. In the female the position of the anus was not found.

The vulva is not salient: it is 1 mm. from the head in one female and 0·78 mm. from the head in a second. The uteri join to form the vagina at some distance from the vulva, and the vagina runs forward from the junction as a wide, thick-walled tube which has a coiled portion just in front of the vulva.

The lateral fields are conspicuous in both sexes and there are large conspicuous hypodermal cells in the tail. The caudal extremity has the two lateral lappets (Fig. 3) characteristic of the genus *Dipetalonema*.

In the male twelve very small caudal papillae are present. They are ventro-

lateral in position, six on each side, but the arrangement is not strictly symmetrical. There are six perianal papillae and three pairs of papillae farther back. Of the perianal group, one papilla on each side is preanal, one on each side adanal and the remaining two are postanal. The papillae of the perianal group are rather larger than those farther back.



Figs. 1–3. *Dipetalonema digitatum*, male: 1, posterior end, side-view showing the large spicule, × 285; 2, posterior end, showing short spicule, × 425; 3, posterior end, ventral view showing two lateral lappets and caudal papillae, × 425.

The large spicule (Fig. 1) is 256 μ long and is divisible into two parts. Proximally there is a strongly chitinized cylindrical portion, but distally the chitinization becomes progressively less pronounced. The tip of the spicule is curved and ends in a double point. The short spicule (Fig. 2) ends bluntly. There are no caudal alae or gubernaculum.

Morphology of the microfilaria (Fig. 4; Table 2)

The microfilariae were studied in the usual way by taking thick blood films, drying them, dehaemoglobinizing and staining with haemalum or Giemsa's stain. They are approximately 200 μ long and 3 μ wide, and are unsheathed. The head space, nerve ring and excretory pore are easily seen, and in many individuals an anal pore is visible. Between the excretory pore and the anal pore there is always

Table 1. *Dimensions of adult worms*

Species ...	*D. sunci*	*D. digitatum*	*D. digitatum*	*D. gracile*
Author ...	Sandground (1933)	Chandler (1929), Sandground (1939)	Author's figures	Author's figures
Host ...	*Suncus coeruleus*	*Hylobates hoolock* and *H. lar. entelloides*	*Macaca speciosa* Monkey 145	*Cebus apella* Monkey 147
Locality ...	Indo-china	India and Siam	India	Brazil
Female				
Length (mm.)	200–230	170–260	194–216	155–165
Max. width (μ)	420	380–550	244–250	
Nerve ring (μ)	250	160		
Oesophagus (μ)		1100–1300	1000	600
Vulva (μ)	600	950–1200	780–1000	700
Tail (μ)	65	330–460		450–580
Male				
Length (mm.)	82–95	100–130	80	83
Max. width (μ)	260	230–270	260	
Nerve ring (μ)	250		128	36
Oesophagus (μ)		1100–1460	864	480
Tail (μ)	100	110–120	116	290
L. spicule (μ)	230–270	250–310	256	980
R. spicule (μ)	100–120	95–120	100·5	217
Spicular ratio	2·2:1	2·6:1	2·5:1	4·5:1

Table 2. *Dimensions of microfilariae*

Monkey ...	134 *Macaca mulatta* (*D. digitatum*)	145 *Macaca speciosa* (*D. digitatum*)	146 *Cebus apella*	147 *Cebus apella* (*D. gracile*)
Total length microfilaria in μ excluding sheath	171 ± 12	205 ± 9	203 ± 9	199 ± 10
Percentage length from anterior extremity to first nucleus of nuclear column	2·7 ± 0·4	2·8 ± 0·5	2·8 ± 0·5	3·0 ± 0·6
Percentage length from anterior end to nerve ring	24·0 ± 1·8	25·6 ± 2·1	22·5 ± 3·3	26·1 ± 1·7
Percentage length from anterior end to excretory pore	35·6 ± 3·8	40·5 ± 3·9	32·3 ± 3·4	34·9 ± 1·6
Percentage length from anterior end to beginning of region of scattered nuclei	49·8 ± 3·4	49·3 ± 3·6	61·0 ± 1·2	62·1 ± 2·3
Percentage length from anterior end to end of region of scattered nuclei	69·1 ± 3·8	74·2 ± 2·8	75·2 ± 2·8	72·0 ± 2·3
Percentage length from anterior end to anal pore	87·7 ± 2·2	—	86·6 ± 0·9	90·2 ± 3·4

a region where the nuclei are fewer in number and more widely spaced than in the rest of the nuclear column. The beginning of this region perhaps corresponds with the 'third break' of some authors. The tail is blunt and usually has a single rather large nucleus at its tip which sometimes results in a slight constriction just in front of this nucleus. Occasionally there are two terminal nuclei. The number of nuclei in single-file in the tail is variable, but there are never more than five and often only one is present.

When microfilariae were examined during life by phase-contrast microscopy, the anterior end of the microfilaria was seen (in its resting position) to be rounded off; at regular intervals, however, a small hemispherical structure surmounted by a short spike was protruded for about half a second and then withdrawn again.

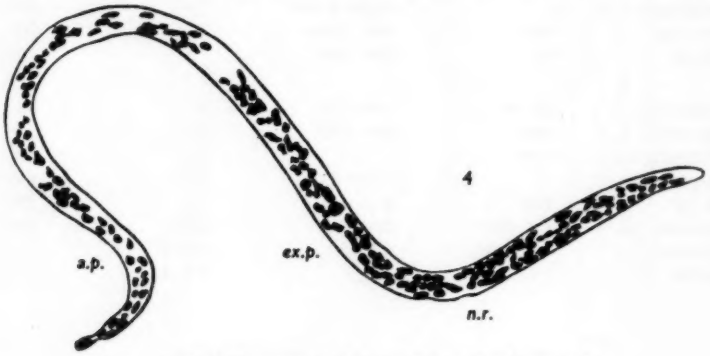


Fig. 4. *Dipetalonema digitatum*. Microfilaria, × 1120.

Systematic position

This worm clearly belongs to the genus *Dipetalonema* Dies. 1861 (= *Acanthocheilonema* Cobbold, 1870). The structure of the long spicule is different from that seen in most species of *Dipetalonema* which have previously been described from primate hosts, but it resembles the structure of the long spicule in *Dipetalonema digitatum* (Chandler). *D. digitatum* was found in gibbons (*Hylobates hoolock*, *H. leucogenys* and *H. lar. entelloides*) in India and Siam (Chandler, 1929; Sandground, 1938). The worms from monkey 145 differ from it only in the number and arrangement of the caudal papillae and the proportions of the left spicule. In *D. digitatum* there is a pair of papillae immediately in front of the caudal lappets, and the remaining post-cloacal papillae are lateral but unpaired. In the worm from monkey 145 the papillae in front of the lappets are absent, but the posterior six papillae are arranged in three pairs. In *D. digitatum* the distal section of the left spicule is longer than the proximal section; in the worm from monkey 145 the proximal section is the longer. Since these differences are slight, it is concluded that the worms from monkey 145 are *D. digitatum*. It is interesting to note also that *D. digitatum* resembles *D. sunci*, a parasite of the insectivore *Suncus coeruleus* from Indo-China (Sandground, 1933). The caudal papillae and spicules of the two species are very similar, but in *D. sunci* the end of the tail is not grooved and the proximal section of the right spicule is longer than in *D. digitatum*.

Life history

(*a*) *Microfilariae.* The microfilariae were periodic and were numerous in the peripheral blood at night and scanty by day. A typical curve is shown in Fig. 5. Evidence has been given in an earlier paper (Hawking & Thurston, 1951) that during the day-time the microfilariae accumulate in the small vessels of the lungs, thus accounting for their scantiness in the peripheral blood. The distribution of the microfilariae in the different organs of the body was determined by post-mortem examination. The monkey died at midnight and 79 % of the microfilariae were

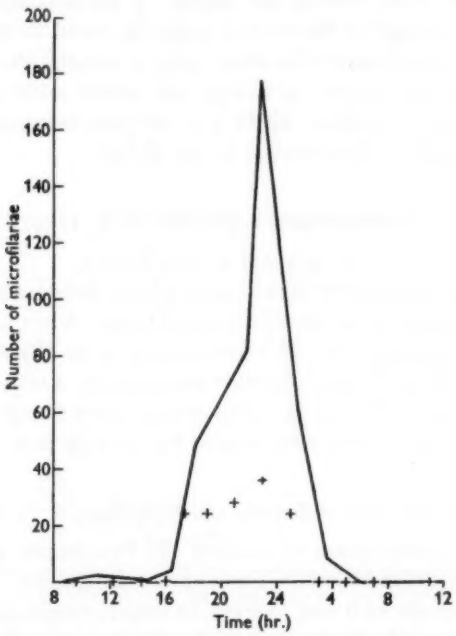


Fig. 5. *Dipetalonema digitatum.* Chart to show periodicity of microfilaria in blood of monkeys 134(+) and 145(——). Ordinate, number of microfilariae, M. 145 mf/10 mm.^{3} blood; M. 134 mf/80 mm.^{3} blood. Abscissa, Greenwich mean time, hours.

in the blood, 10·6 % in the lungs, 3 % in the liver, 0·4 % in the spleen, 3·4 % in the skin, 2·7 % in the heart. Other organs contained only negligible numbers.

During the period of observation (3 months) there was no significant change in the maximum number of microfilariae in the blood, although the cycle was partially inverted on several occasions, presumably owing to disturbance of the sleeping habits of the monkey.

(*b*) *Vectors.* At various times when the blood contained numerous microfilariae, mosquitoes were fed on the monkey and dissected after 7 or more days. No development of the larval filariae was detected in 55 *Aëdes aegypti*, 7 *A. albopictus*, 6 *Anopheles quadrimaculatus* or 14 *An. stephensi*. Probably, therefore, none of these mosquitoes is capable of transmitting this species of filarial worm.

(*c*) *Transplantation*. After the death of monkey 145, six females and one male worm were transferred with sterile precautions to the peritoneal cavity of a *Macaca mulatta* (no. 134) on 16 July 1948. No microfilariae were found in the blood of monkey 134 during night examinations up to and including 8 September 1948 (10.0 p.m.) but they were discovered (ten microfilariae per 20 mm.^{3}) on 1 October 1948, at 11.30 p.m., i.e. 2½ months after insertion of the adult worms. They showed the nocturnal periodicity as illustrated in Fig. 6, which relates to 19 November 1948. They were seen on 25 January 1949, when there were five microfilariae per 20 mm.^{3} at 5.30 p.m., but they were absent on 1 March 1949, when successive examinations were made during the night. A laparotomy was performed on 29 July 1949, but no sign of the worms could be found in the peritoneal cavity. Thus microfilariae were found in the blood up to 6 months after the insertion of the adult worms; it is not known how long the adults survived, but presumably they lived for at least 3 months, which was the time required for microfilariae to accumulate sufficiently to be detected in the blood.

Dipetalonema gracile (Rud. 1809)

Origin and general history

Twelve *Cebus apella* monkeys were obtained from Brazil near Rio de Janeiro and microfilariae were found in the blood of three of them. Adult worms were recovered from one of these monkeys (no. 147) and proved to be *Dipetalonema gracile*. The microfilariae in the blood of one of the other monkeys (no. 146) were indistinguishable from those of monkey 147, but no adult worms were found in the monkey. No detectable pathological lesions were caused by the infection.

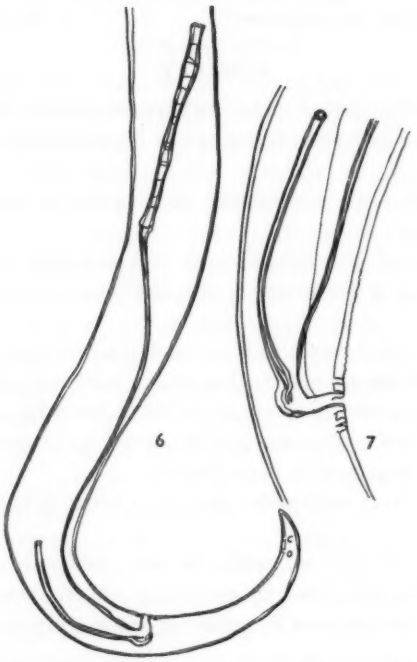
Morphology and systematic position (Figs. 6, 7; Table 1)

At post-mortem examination of monkey 147 two female worms and one male were found in the peritoneal cavity lying on the mesentery.

The female worms are 15·5 and 16·5 cm. in length, respectively. Anteriorly, the worms do not taper markedly, so that the head is broad, and it has a squared outline. Four cephalic papillae are easily seen. The mouth, which is not provided with lips, opens into the oesophagus which is about 600μ long; the division between oesophagus and intestine is not very sharp. The uteri join to form the vagina some distance from the anterior end. The vagina runs forward to a point in front of the vulva and then curves round to open on the surface at a distance of 700μ from the head. The anus is 450–580μ from the posterior extremity. There is one pair of subterminal caudal appendages.

The single male worm is 8·3 cm. long. The head is similar to that of the female. The oesophagus is 480μ long and is encircled by the nerve ring at a distance of 36μ from the head. The cloaca opens 290μ in front of the caudal extremity. There is a pericloacal group of five pairs of small sessile papillae (Fig. 7). Towards the end of the tail there are two further pairs (Fig. 6) and there is a subterminal pair of petal-shaped papillae. The spicules are very unequal in length, the longer being 980μ and the shorter 217μ, and the ratio between their lengths is therefore 4·5:1. The

long spicule has a strongly thickened proximal part 290μ long, so that the ratio of the lengths of the proximal and distal sections is 1:2·3. The distal part of the spicule is very narrow and reaches almost to the level of the cloacal orifice. The short spicule is cylindrical proximally but not so heavily thickened as the long spicule; distally it ends in a hook.



Figs. 6–7. *Dipetalonema gracile*, male: 6, tail showing spicules and subterminal pair of papillae, × 113; 7, tail showing the right spicule and pericloacal papillae, × 233.

The cuticle in both sexes is smooth with transverse striations.

The microfilariae (Table 2) are indistinguishable morphologically from those of *Dipetalonema digitatum* (see above).

These worms agree with previous descriptions of *Dipetalonema gracile* (Rud.), reviewed in Webber (1955).

Life history

(*a*) *Microfilariae.* The number of microfilariae in the blood of both monkey 146 and monkey 147 fluctuated but it showed no definite cycle.

The number of microfilariae in the blood of monkey 147 varied from 2 to 21 per 20 mm.^{3} over a period of 14 months until the time of its death, but no upward or downward trend was discernible. In the blood of monkey 146, up to ten microfilariae per 20 mm.^{3} were found over a period of 1 yr. Subsequent films revealed few or no microfilariae and the last occasion on which they were found was 3 years after they had first been seen.

Post-mortem examination of monkey 147 showed that 53% of the microfilariae were in the blood, 30% in the muscles, 16% in the lungs, and negligible numbers elsewhere (Hawking & Thurston, 1951).

(*b*) *Vectors.* During May, June and July 1949 batches of mosquitoes were fed on monkey 147 and dissected for developing filariae 10 days after feeding. No worms were found in 200 *Aëdes aegypti*, 160 *A. albopictus*, 200 *Anopheles maculipennis atroparvus*, and 40 *An. stephensi*.

SUMMARY

1. Filarial worms were found in the peritoneal cavity of a monkey (*Macaca speciosa*) from Bengal. They were identified as *Dipetalonema digitatum* (Chandler, 1929).

2. The microfilariae were unsheathed and showed a well-marked nocturnal periodicity in the blood.

3. No development of the microfilariae was detected in *Aëdes aegypti*, *A. albopictus*, *Anopheles quadrimaculatus* or *An. stephensi*, but only a few mosquitoes were observed.

4. Six female worms and one male were transplanted into the peritoneal cavity of a rhesus monkey (*Macaca mulatta*). Ten weeks later microfilariae were found in the blood of the monkey and they persisted for at least 16 weeks.

5. Filarial worms (*Dipetalonema gracile*) were discovered in the peritoneal cavity of a monkey (*Cebus apella*) from Brazil.

6. The microfilariae were unsheathed and were found in the blood. They showed no periodicity.

7. No development of the microfilariae was detected in *Aëdes aegypti*, *A. albopictus*, *Anopheles maculipennis atroparvus* or *An. stephensi*.

8. No pathological lesions due to either of these worms were detected in the monkeys.

We wish to thank Mr P. Sewell, Ph.D., for help in the identification of the filarial worm from the macaque monkey, and Mr E. C. England for technical assistance.

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THE LOUSE POPULATIONS OF SOME CRICETID RODENTS*

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(With 4 Figures in the Text)

INTRODUCTION

An investigation of the literature on the sucking lice, which has been well summarized by Hopkins (1949), reveals that we have very little knowledge of their biology. While a considerable body of information is available on the human louse, and a small amount is available on a few of the lice of domestic animals, next to nothing is known concerning the ecology of lice infesting wild hosts under natural conditions. Most of the data that are available on louse populations of wild hosts are based on very small host samples and on parasite samples recovered by the use of techniques inadequate for quantitative work. A few papers contain population data in regard to lice which were derived incidentally from studies of flea populations in relation to murine typhus and sylvatic plague. We are familiar with but one paper directed toward an understanding of the bionomics of louse populations on a wild host (Vysotskaia, 1950). This paper is based on a study of grey voles (*Microtus arvalis*) collected in an area near Leningrad, U.S.S.R. While the data presented in this paper are extensive, there is no discussion of the methods used, and the data cannot be properly evaluated. This is true of most other studies on louse populations. Only two or three papers indicate any precautions taken to prevent transfers of lice from one host to another between the time of capture and examination. Most investigators have relied on searching, brushing or some undescribed vacuum device for recovery of lice. We do not believe that any of these are adequate for the recovery of all stages nor that all adult lice can be recovered thereby. The techniques that we have used to prevent contamination and to recover the total louse population have been described (Cook, 1954*a*, *b*), but they are reviewed here briefly.

To prevent transfers of lice from one host specimen to another and to reduce loss, each specimen is placed in a separate one-pint size plastic home food freezer bag immediately on recovery from a trap. Each bag is numbered, and each skin is returned to this original bag after the host is examined, skinned and the skin dried. Care is used during examination and skinning to prevent loss of lice. The recovery of all the lice is accomplished by dissolving the whole skin with trypsin followed by KOH. The lice are then strained out of the resulting solution. This recovers all stages including embryos sufficiently developed to have acquired a chitinous exoskeleton.

The present study grew out of an investigation of population fluctuations in the small mammals in the Basswood Lake area of northern Minnesota (Beer, Lukens & Olson, 1954). Beginning in 1952 the small mammal specimens collected during this survey were saved to facilitate the study of their ectoparasite populations.

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SPECIES OF LICE PRESENT

This report is concerned with the two species of Anoplura found on the three species of cricetid rodents available in adequate numbers in the study area. These are: *Hoplopleura hesperomydis* (Osborn) which occurs on the deer mouse, *Peromyscus maniculatus* (Wagner), and *Hoplopleura acanthopus* (Burm.) which occurs on the meadow vole, *Microtus pennsylvanicus* (Ord), and the redback vole, *Clethrionomys gapperi* (Vigors), in the Basswood Lake area. We have examined 798 skins from the three hosts mentioned (Table 1). These were taken between 21 June and 23 July in 1952 and 1953.

Table 1. *Host specimens examined*

Host	1952	1953	Total
Peromyscus maniculatus	105	275	380
Microtus pennsylvanicus	155	76	231
Clethrionomys gapperi	125	62	187
Total	385	413	798

At present only the two previously recorded species of *Hoplopleura* have been recovered. The lack of any *Polyplax* is rather puzzling since *P. abscisa* Fahrenholz has previously been recorded from the meadow vole in New York (Ferris, 1951) from the California vole (*Microtus californicus*) and the sage brush vole (*Lagurus intermedius*) from Nevada (Ferris, 1951). *Polyplax alaskensis* has been recovered from the tundra mouse (*Mus operarius*) in Alaska (Quay, 1949) and from the large-toothed redback vole (*Clethrionomys rufocanus*) in Europe (Ferris, 1951). Vysotskaia (1950) records *Polyplax serrata* (Burm.) which is normally a parasite of the house mouse (*Microtus musculus*) and *Polyplax* sp. from the grey vole (*Microtus arvicola*) near Leningrad but indicates that they are very rare and that *Hoplopleura acanthopus* is the dominant species. *Polyplax auricularis* is recorded from the deer mouse in California, from the sitka mouse (*Peromyscus sitkensis*) in Alaska (Ferris, 1951) and from the California mouse (*P. californicus*) in California (Holdenried, Evans & Longenecker, 1951). The apparently discontinuous distribution of these species of *Polyplax* is curious when one considers that the ranges of two of the hosts, the deer mouse and the meadow vole, cover most of North America. Both *Hoplopleura acanthopus* and *H. hesperomydis* have been widely collected in North America from these hosts. This suggests that the parasite distribution is not necessarily coincident with the distribution of its host.

HOST SPECIFICITY

Host specificity has been generally assumed, but there is little concrete data bearing on the subject. Jameson (1947) has indicated that only *H. acanthopus* was found on the prairie vole (*Microtus ochrogaster*) during a study conducted in the vicinity of Lawrence, Kansas, despite the fact that the cotton rat (*Sigmodon hispidus*) and two species of *Peromyscus* used the same runs. The cotton rat harboured only *Hoplopleura hirsuta* and *Peromyscus* spp. only *Hoplopleura hesperomydis*. Worth

(1950), working in Florida, found the same situation in the case of the cotton rat which was parasitized by *H. hirsuta* and the rice rat (*Oryzomys palustris*) which was parasitized by *Hoplopleura quadridentata*. Data presented by Morlan (1952) in regard to *Polyplax spinulosa* which he found on two species of *Peromyscus* as well as the normal hosts, the black rat (*Rattus rattus*) and the Norway rat (*R. norvegicus*), appear to contradict the assumption that the lice are host specific. These records seem, however, to be cases of contamination.

Table 2. *Rate of infestation with lice in per cent*

		Hoplopleura hesperomydis		*H. acanthopus*	
Host	Sex	1952	1953	1952	1953
Peromyscus maniculatus	Male	58·0	18·5	6·0	0
Peromyscus maniculatus	Female	25·0	11·4	0	0
Average		41·0	15·0	—	0
Microtus pennsylvanicus	Male	0	0	58·0	91·5
Microtus pennsylvanicus	Female	1·2	0	66·5	78·0
Average		—	0	62·0	83·0
Clethrionomys gapperi	Male	0	0	3·2	0
Clethrionomys gapperi	Female	0	0	7·9	2·9
Average		0	0	5·6	0·9

It is evident from the parasites collected at Basswood Lake that transfer of lice from their normal host species to other species is extremely rare, despite the fact that these hosts occupy the same general area and often the same habitat and may even use the same runs. Of the lice collected from deer mice 1003 of 1007 or 99·7% were *Hoplopleura hesperomydis*. Of those collected from meadow voles 2793 of 2797 or 99·9% were *H. acanthopus*. Of those taken from redback voles 13 of 13 were also *H. acanthopus*. The rate (percentage) of infestation by species is shown in Table 2.

It is worth mentioning that the rare collection of lice on other than the normal host occurred only in the first year's samples. It was discovered at the end of the first year's work that one possible source of contamination had been overlooked—the table on which the hosts were examined. A change in technique to reduce this error was introduced in 1953. The lack of abnormal associations in the 1953 sample suggests that the abnormal associations in 1952 were contamination rather than straggling.

RATE OF INFESTATION

Table 2 gives the percentage of hosts infested with lice in 1952 and 1953. *H. hesperomydis* is considered to be the normal parasite on deer mice and *H. acanthopus* the normal parasite of meadow voles and redback voles. The following discussion will deal strictly with what we consider to be normal host-parasite relationships. There is considerable difference in the rate of infestation between the two years' samples of each host. The rate of infestation in deer mice was considerably higher

in 1952 than in 1953, 41 % as compared to 15 %. The infestation rates also varied in meadow voles but in the opposite direction, 62 % as compared with 83 %. There was variation noted in the redback vole collections, but the number of infested animals was so small that it is difficult to evaluate the variation. The wide difference in rate of infestation in the two summers' samples may indicate year to year fluctuations of these magnitudes or may indicate that the two collections, while made during the same period each year, were not made during phenologically comparable periods.

Table 3. *Relative density of small mammal populations as based on trapping success*

	Captures per 1000 trap days			
Species	1950	1951	1952	1953
Peromyscus maniculatus	4·3	22·0	22·4	71·9
Microtus pennsylvanicus	7·2	5·2	36·2	29·8
Clethrionomys gapperi	4·3	21·5	29·0	17·4

During the 4 years that the host populations were under observation, considerable yearly difference was noted in their density (Table 3, Beer *et al.* 1954). The deer mouse had a very low population density in 1950 and showed considerable increase in 1951. In 1952, the first year of the parasite study, the host population showed little change from that of 1951, while in 1953 there was a tremendous increase in the host population density. In 1952 the infestation rate was 41 %, while in 1953 the infestation rate was only 15 %. The meadow voles showed a very low population density in 1950 and 1951 with a sharp rise in 1952 followed by a general drop in 1953. The rate of infestation by lice was 62 % in 1952 and 83 % in 1953. The number of infested redback voles was so small that a comparison on this basis has no significance.

One fact is worth noting in regard to the difference in percentage of infestation between the two years' samples. The higher percentage of infested deer mice occurred in 1952 when the population was relatively stable while the lower observed rate occurred in 1953 when the population of mice had increased (Tables 2 and 3). In contrast, the higher rate of infestation in meadow voles occurred in 1953 (83 %) rather than in 1952 (62 %) concurrent with a declining host population. These data suggest the possibility that the infestation rate is higher in a declining host population and lower in a rapidly increasing population. However, variations as great as these have occurred within 2 months in the case of *Neohaematopinus neotomae* (Ferris) on the dusky-footed wood rat (*Neotoma fuscipes*) as shown by Linsdale & Tevis (1951). Vysotskaia (1950) also records comparable infestation rate fluctuations within 2 months in *Hoplopleura acanthopus* on the grey vole. Comparison of the rates of infestation in the same months of 1941 and 1945, as given by the latter author, reveal little significant difference.

There are few other infestation rates available in the literature for comparison. Jameson (1947) found an infestation rate of 56 % in the 59 prairie voles he examined between October 1945 and August 1946. The few species of *Peromyscus*

that have been examined by other workers show very low infestation rates. Harkema (1936) examined 54 white-footed mice over part of 1 year and found no lice whatsoever. Royal (1952) examined 42 white-footed mice during 1 year and found 4 mice infested with a total of 5 lice. Morlan examined 249 cotton mice and 243 old fieldmice over a period of 5 years and found 13 and 1 of these respectively infested. The infestation rates derivable from these figures are much below that for the deer mice examined by us. The differences are probably largely due to inadequate recovery techniques so that comparisons are not justified.

Table 4. *Average total louse population by year, host species and sex of host*

		1952		1953	
Host species	Sex of host	Average no. per infested specimen	Average no. per specimen examined	Average no. per infested specimen	Average no. per specimen examined
Microtus pennsylvanicus	Male	24·0	15·1	29·8	27·3
Microtus pennsylvanicus	Female	14·1	9·2	6·4	5·0
Peromyscus maniculatus	Male	16·7	9·7	8·1	1·5
Peromyscus maniculatus	Female	13·4	3·4	7·5	0·9

Differences in infestation rate between male and female hosts are also shown in Table 2. A striking difference can be observed between the rate of infestation in males and females of deer mice in both summers with the males showing the higher rates, 58 and 18·5% as compared to 25 and 11·4%. In addition, the male deer mice had a larger average louse population than did the females (Table 4). The louse population in meadow voles is usually greater per individual in the males than in the females, but the rate of infestation is not always greater. In 1952 a larger percentage of females were infested than males, while in 1953 the reverse was the case. Worth (1951) has noted heavier infestation rates in the males of cotton rats from an area in Florida. He found 22·2% of the males were infested by lice and only 3·4% of the females. Worth attributes this difference to the greater mobility of the males and the more frequent contact made by them with other hosts.

When the data were examined to determine the possible effect of age of host on rate of infestation, no correlations were found. The rate of infestation appears to be independent of the age of the host.

The difference in infestation rates among species is shown in Table 2. These differences are even apparent between host species that harbour the same species of louse. Both meadow voles and redback voles have been recorded in the literature as being hosts of *Hoplopleura acanthopus*. The rate of infestation recorded by us for redback voles was so low that we felt there was a possibility that it might be contamination. However, we have examined one specimen of this host from the Cloquet Forest Experiment Station, Carlton County, Minnesota, which bore eggs, nymphs and adults. This host was taken without any possibility of contamination from other hosts. This suggests that the infestations found on this host in the

Basswood Lake area were normal. The marked difference in infestation rates of these two hosts, 62 and 83 % in meadow voles and 5·6 and 0·9 % in redback voles, cannot be explained at present. It may be that the populations of lice on these two hosts are independently variable and that our samples were taken from a low point in the seasonal fluctuation on redback voles and from a high point on meadow voles. A full year's sample from each host must be collected before any definite conclusion can be reached.

Population differences between two species of lice are also noted (Tables 2 and 4). *H. hesperomydis* occurs with a lower frequency and in smaller numbers on the deer mice than does *H. acanthopus* on meadow voles, although at a higher frequency than on redback voles. From our samples, the fluctuations in the populations also appear to be independent of each other in spite of the fact that both hosts were taken from the same area at the same time. Apparently the louse population depends upon the characteristics of the host population. This would seem to rule out climatic factors as being direct controlling forces in determining either rate of infestation or louse population density.

LOUSE POPULATION STRUCTURE

To our knowledge there have been no previous attempts to determine the relative numbers of each of the early instars in the louse populations on wild mammals. Some data have been published by Hopkins (1949) and by MacLeod & Craufurd-Benson (1941) on adult:nymph ratios in the human louse. These recorded ratios varied from 1:2·2 to 1:3·6. Vysotskaia (1950) indicates that the proportion of nymphs in the population of *H. acanthopus* on grey voles varies seasonally. He suggests that this may be correlated with the life cycle of the louse (which is unknown) and with such extrinsic factors as host moulting and nest digging activities.

Table 5. *Population structure of* Hoplopleura acanthopus *found on* Microtus pennsylvanicus *in* 1952 *and* 1953

Sex of host ...	Males				Females					
Year ...	1952		1953		1952		1953		Total	
No. of hosts with lice ...	43		22		53		38		156	
Lice	No.	%	No.	%	No.	%	No.	%	No.	%
First instar	443	39·0	240	36·4	281	37·4	19	7·9	983	35·2
Second instar	173	15·2	93	14·1	121	16·1	10	4·1	397	14·2
Third instar	54	4·6	29	4·4	30	4·0	3	1·2	116	4·2
Adult males	185	16·3	134	20·3	120	16·0	90	37·2	529	19·0
Adult females	281	24·7	164	24·8	199	26·5	120	49·6	764	27·4
Total	1136	—	660	—	751	—	242	—	2789	—

The population structures of the lice on infested meadow voles and deer mice examined by us are given in Tables 5 and 6. These are the actual numbers of lice recovered in each of the various categories, and the proportion of each life stage found in the sample. It is apparent that in both *H. acanthopus* and *H. hespero-*

mydis the adult to nymph ratio, while in favour of the nymphs, is not far from 1 to 1. This is at variance with the ratios that have been cited in the literature.

It is further apparent that the proportions of the various instars of the ectoparasites in the 2 years' samples are relatively consistent in the two species concerned, despite the change in infestation rate and population density. It is true that the proportion of adults to nymphs increased considerably in the case of the lice of the female meadow voles in 1953, while the lice of the deer mouse, on the other hand, show a larger proportion of adults to nymphs in the male hosts in 1953. The proportion on the female hosts is relatively constant for the 2 years. The difference apparent in both cases might be attributed to sampling deficiencies.

Table 6. *Population structure of* Hoplopleura hesperomydis *found on* Peromyscus maniculatus *in* 1952 *and* 1953

Sex of host ...	Males				Females					
Year ...	1952		1953		1952		1953		Total	
No. of hosts with lice ...	29		25		14		16		84	
Lice	No.	%	No.	%	No.	%	No.	%	No.	%
First instar	209	43·2	67	33·0	86	45·7	43	35·5	405	40·7
Second instar	62	12·8	6	3·0	16	8·5	13	10·7	97	9·7
Third instar	30	6·2	8	3·9	3	1·6	6	5·0	47	4·7
Adult males	93	19·2	52	25·6	36	19·1	26	21·5	207	20·8
Adult females	90	18·6	70	34·5	47	25·0	33	27·3	240	24·1
Total	484	—	203	—	188	—	121	—	996	—

Attention should be called to the progressive decrease in numbers of specimens of second instars in comparison to numbers of first, and third instars compared to second. This decrease is very consistent in both host species examined. It is suggested that nymphal mortality accounts for this regular decrease in numbers of successive instars. The greater duration of the adult stadium in the lice would account for the larger proportion of these in the population at any one time.

Female lice are more numerous than males in both species in the 2 years' samples, although this predominance is not great. This might be accounted for by a shorter life span for the male louse. The latter view has been advanced in regard to one species of cattle louse by Craufurd-Benson (1941).

Table 7. *Percentage of infested hosts with mature embryos*

	1952		1953	
	Male mice	Female mice	Male mice	Female mice
Hoplopleura acanthopus	42	37	81	26
H. hesperomydis	8·7	8·3	0	0

Table 7 indicates the percentage of infested hosts from which embryos were recovered. A very wide difference is apparent here between the lice of the meadow vole and those of the deer mouse. The eggs of both of these species are nearly the

same size, and the recovery technique was identical. Despite this, only a small percentage of deer mice were found to harbour eggs* in 1952, and none were found in 1953. A large percentage of infested meadow voles were found to harbour eggs in both years. It has been suggested by Vysotskaia (1950) that reproductive activity in the lice infesting the grey vole in Russia is cyclic. This might explain the difference between these two parasite species if the lice of the deer mouse and those of the meadow vole have different cycles. This rather contradicts what we have found in the case of the nymphs where a fairly constant ratio between first, second and third instars is apparent. The latter would indicate a constant reproductive activity—at least during the periods sampled. It must be admitted that the egg sample has been small, and the differences may not be real.

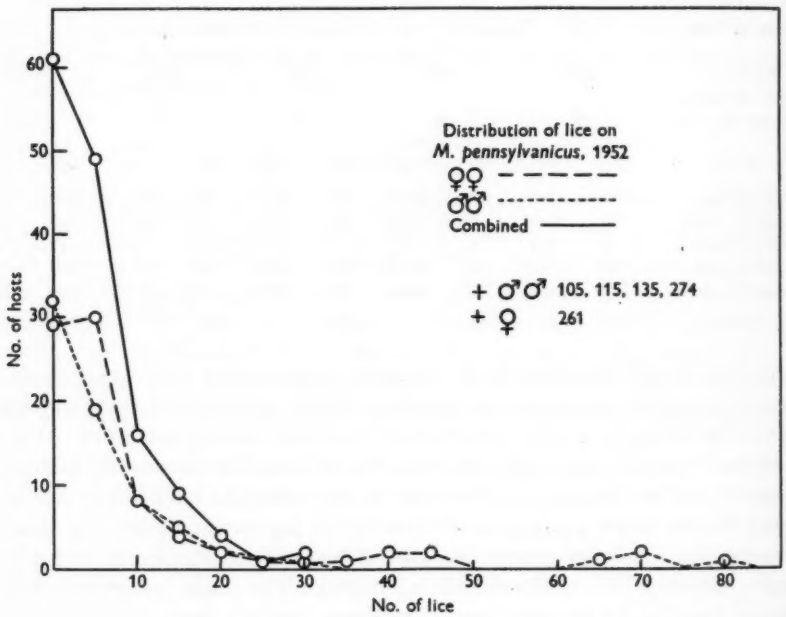

Fig. 1.

SIZE OF LOUSE POPULATIONS

Several previous workers have given some indication of population size on infested hosts by either giving the mean number of lice per infested host specimen or per host examined or have given maximum and minimum numbers of lice found. Morlan (1952) found that the cotton mice he examined harboured 8·2 lice per infested specimen. Jameson (1947) found that the prairie voles he examined had 6 lice per infested specimen. Vysotskaia (1950) found that the grey voles he

* Eggs as such are not recovered by the technique used, but embryos that have developed far enough to have a chitinous cuticle are recovered. These are quite distinctive from first instars.

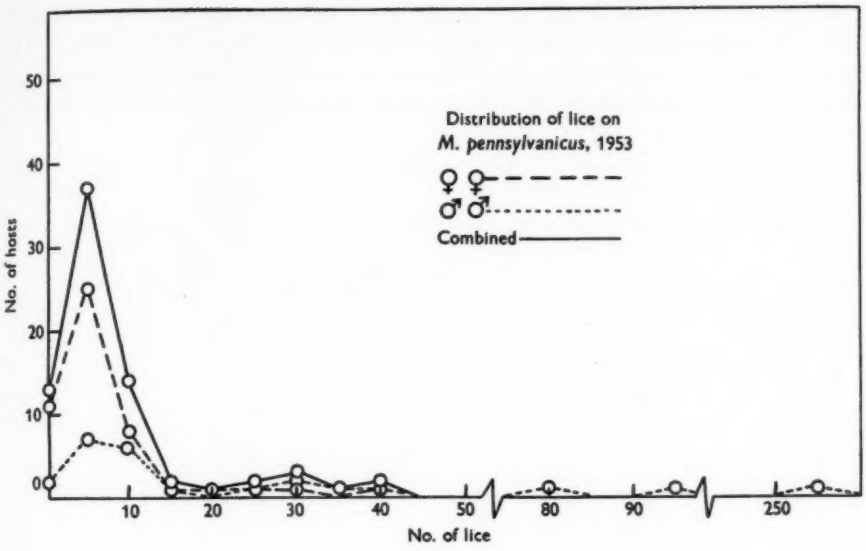
Distribution of lice on
M. pennsylvanicus, 1953
♀♀
♂♂
Combined
No. of hosts
No. of lice
0
10
20
30
40
50
10
20
30
40
50
80
90
250

Fig. 2.

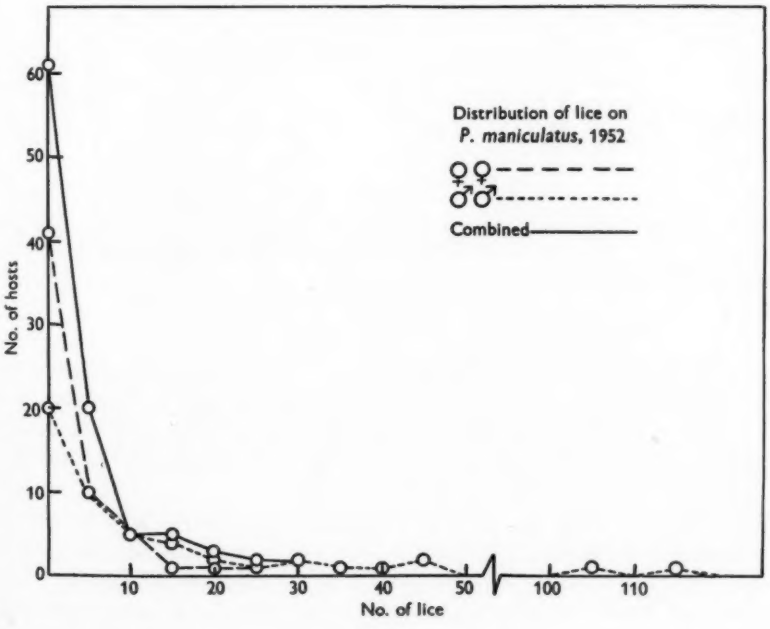
Distribution of lice on
P. maniculatus, 1952
♀♀
♂♂
Combined
No. of hosts
No. of lice
0
10
20
30
40
50
60
10
20
30
40
50
100
110

Fig. 3.

examined in 1941 had 21·8 and in 1945 an average of 26·8 lice per infested host. Our figures (Table 4 and Figs. 1–4) for *H. acanthopus* on meadow voles are much closer to those recorded by Vysotskaia (1950) than to those of Jameson (1947). In the case of *H. hesperomydis* on deer mice our average infestation rates are all fairly close to those derivable from data reported by Morlan (1952) for cotton mice.

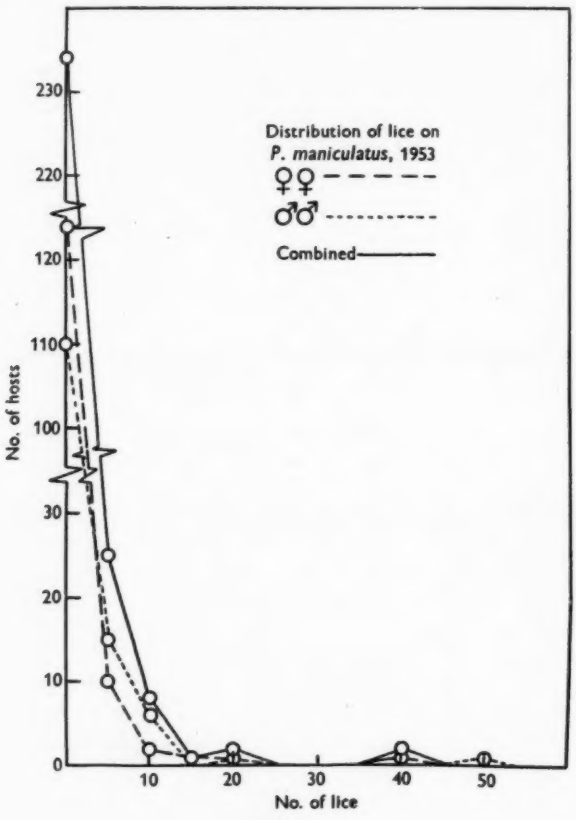

Fig. 4.

It should be noted that the averages given in many cases are badly skewed by a small number of hosts which harbour very large louse populations. This has been discussed by Cole & Koepke (1946). The size of the populations on individual hosts is best comprehended by reference to Figs. 1–4 where the number of lice per host is plotted against the number of hosts examined.

It has been suggested that the size of the louse population may be affected by the age and sex of the host. Our data show that the males harbour larger average populations of lice than do the females (Table 4). This was especially true of the meadow voles where the males averaged 24·0 and 29·8 lice as compared with 14·1 and 6·4 lice per female for 1952 and 1953 respectively. While the same trends appear to hold with deer mice, the differences are not pronounced.

SUMMARY

In 1952 and 1953, 798 specimens of *Peromyscus maniculatus*, *Microtus pennsylvanicus* and *Clethrionomys gapperi* were examined for total louse populations. Two species of sucking lice, *Hoplopleura acanthopus* and *H. hesperomydis*, were found on these species. *H. acanthopus* was found almost exclusively on *Microtus pennsylvanicus* and *Clethrionomys gapperi*, and *Hoplopleura hesperomydis* was found almost exclusively on *Peromyscus maniculatus*.

Contamination appears to account for the apparently abnormal associations.

The rates of infestation varied from host to host and from year to year. In general the higher infestations were found on host populations which were stable or declining, and the lower rates were on hosts which were increasing in density. *Microtus pennsylvanicus* had the highest infestation rate followed by *Peromyscus maniculatus*, with *Clethrionomys gapperi* nearly free of lice.

The age of the host apparently had little to do with rate of infestation or population size.

The louse populations were made up of about equal numbers of adults and nymphs. The adult sex ratio was, in each sample, unbalanced in favour of the females.

The average population size varied between sexes of host and years. The male hosts had a higher average population than the female.

This work was partially supported by the Quetico-Superior Wilderness Research Station. Mr David Olson, Mr Norman Hansen and Mr Daniel Frenzel helped to collect the mouse skins used for this study. Dr M. Haydak translated the Russian paper by Vysotskaia.

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OBSERVATIONS ON THE STRUCTURE AND THE NATURE OF '*NR* BODIES' FOUND IN THE TESTES OF BED-BUG

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(With Plate VI)

INTRODUCTION

Hertig (1936) described, as constant feature of the gonad of *Culex pipiens*, large cell-inclusions, which he termed '*NR* bodies', on account of their affinity for neutral red. These were composed usually of granules and rods embedded in a matrix. These cell-inclusions superficially resembled masses of rickettsiae on the one hand and inclusion bodies of certain viruses on the other. Hertig considered the rickettsiae, which were also found affecting the gonads, and the *NR* bodies, to be apparently unrelated. The appearance and behaviour of *NR* bodies seemed to be consistent with their being living agents or the manifestations thereof. They were associated with a pathological condition which brought about destruction of primary ovarian follicles.

Hertig also mentioned that a brief examination of a few specimens of *Cimex lectularius* and *C. pipistrelli* showed that the gonads and the mycetomes contained a number of bodies that stained with neutral red. They were not, however, studied further.

While examining the gonads of *C. lectularius columbarius* Jen. and *C. lectularius lectularius* Linn., collected at Mukteswar-Kumaun and at Calcutta, we have come across bodies stainable with neutral red, which simulate closely the *NR* bodies of *Culex pipiens*, described by Hertig (see Ray & Dasgupta, 1954). The male gonads were always found to be infected with them, but they were rare in the female gonad. Further study of the female gonad and the mycetomes is in progress, but we submit here a preliminary report on the structure and the nature of the bodies that stained with neutral red, and which were found to be causing heavy infection of the testes of these bugs.

MATERIAL AND METHODS

The testes were dissected out in normal saline solution to which a little neutral red was added. The different forms of the *NR* bodies took up the stain in 10–15 min. and were studied under the microscope. They were also studied, under the phase-contrast microscope, in saline to which no neutral red had been added. For sectioning, the testes were dissected out in saline and fixed in alcohol-formalin, in Carnoy's fluid and in Serra's fluid. They were embedded in 'Tissuemat' for 2 hr. at 58° C. and were cut 5–7μ thick.

* Part of this work was done at the Indian Veterinary Research Institute, Mukteswar-Kumaun, U.P. (India), before the authors went to their present address.

For a general study of the morphology of the *NR* bodies, tissues fixed in alcohol-formalin were stained with iron-haematoxylin.

Tissues fixed in Serra's fluid were stained with toluidine blue for study of the distribution of nucleic acids in the *NR* bodies.

Tissues fixed in Carnoy's fluid, and also those fixed in Serra's fluid, were stained with Unna-Pappenheim and were also subjected to the Feulgen technique to study the distribution of DNA and RNA in the body of the parasite.

To study the distribution of polysaccharides in the *NR* bodies, testes freshly dissected out were treated with Lugol's iodine. Materials fixed in alcohol-formalin were subjected to the Bauer-Feulgen technique described by Bensley (1939), while those fixed in Carnoy's fluid and also in Serra's fluid were subjected to the Hotchkiss technique (Glick, 1949) and also to the ammoniacal silver nitrate technique described by Jarvi & Levanto (1950). Material treated with Carnoy's fluid was also subjected to Hale's method (1946) to demonstrate the hyaluronic acid type of polysaccharide in the *NR* bodies. Gomori's revised method (Glick, 1949) was employed for detecting alkaline phosphatase in these bodies.

OBSERVATIONS

NR *bodies as seen in neutral red preparations*

(Pl. VI, fig. 1)

Within 10–15 min. after the addition of this dye, the majority of the *NR* bodies took up the stain, but the stain showed signs of fading away after 4 hr. These bodies appeared as more or less rounded structures with irregular outlines and their size varied between 0·25 and 12·5μ in diameter. All the bodies in a group did not stain with neutral red with equal intensity. Those which took up the stain appeared as granulated masses. Smaller *NR* bodies were seen to bud off from the larger ones. Minute *NR* bodies were seen to lie in the cytoplasm of the infected spermatogonial cells and sometimes were seen to be situated on the outer surface of the nuclear membrane. Such forms were very minute and could only be seen by careful observation. Groups of mature *NR* bodies were often found to be enclosed within healthy sperm-packs. Larger *NR* bodies could be recognized in saline preparations without the aid of neutral red.

Phase-contrast appearance of NR *bodies*

(Pl. VI, fig. 5)

Under the phase-contrast microscope mature *NR* bodies appeared very bright and refractile. Some of them, which were either not mature or were undergoing repeated budding, showed dark granules. Two or three *NR* bodies were also seen to be enclosed within a common envelope. Very minute spherules appeared as vacuoles with one to four dark dot-like areas at the periphery. In a few cases such single spherules were seen to be lodged in the cytoplasm of the spermatogonial cells. 'Pathological cells' (see below) showed irregular nuclei interspersed with light and dark areas. In some of these cells no nuclear structure could be recognized.

NR *bodies as seen in fixed preparations*

(Pl. VI, fig. 2–4)

In sections, and also in smears, *NR* bodies were always found to be perfectly spherical in form. In specimens stained with iron-haematoxylin the entire mass of *NR* bodies took up a deep black stain with less strongly staining vacuolations. The minute spherules also showed a distinction between a clear area and a deeply stained area. *NR* bodies stained blue with toluidine blue and the picture appeared more or less like that obtained with iron-haematoxylin, with less strongly stained vacuolations. In very minute spherules there appeared to be only a single vacuole, with, on one side of the spherule, deep blue material, crescentic in outline.

With Unna-Pappenheim stain the *NR* bodies showed a very prominent pyronin-staining cytoplasm. This could be easily distinguished from the cytoplasm of the spermatogonial cells. A variable number of globules that stained with methyl-green were found to be embedded in the pyronin-staining matrix of the *NR* body. Some of the *NR* bodies showed minute bud-like forms, which were seen to fall off and give rise to minute spherules which took up the pyronin stain intensely. Such spherules were often seen to migrate towards fresh spermatogonial cells.

With the Feulgen technique DNA was seen to be distributed in the larger *NR* bodies in the form of small globules or dots, while the rest of the body was stained a uniformly light green by the counter-stain. The minute spherules showed a prominent DNA dot with a very small amount of material that took up the counter-stain. It was not uncommon to come across such minute spherules embedded in the cytoplasm of healthy spermatogonial cells, or attached to the outer surface of the nuclei of the spermatogonial cells. Spermatogonial cells containing such spherules often showed degeneration of their nuclei. Such cells may be compared with cells in the female gonad of *Culex pipiens* that have been called by Hertig (1936) 'pathological cells'. In our preparations we have come across a group of 'pathological cells' surrounded by healthy spermatogonial cells. It seems quite evident that while the unaffected spermatogonial cells develop into mature sperms, the affected cells were converted into *NR* bodies.

Polysaccharides in NR *bodies*

Lugol's iodine failed to detect any trace of glycogen in the *NR* bodies. This was confirmed by subjecting the sections to the Bauer–Feulgen technique (Bensley, 1939). With the Hotchkiss method (Glick, 1949) the *NR* bodies revealed the presence of polysaccharides in the form of granular red dots embedded in a lightly pink stained matrix. With the ammoniacal silver nitrate technique described by Jarvi & Levanto (1950), the *NR* bodies also confirmed the presence of polysaccharides in the form of discrete dark-brown granular bodies in a lightly stained matrix. The polysaccharides present in the *NR* bodies were demonstrated, by treatment with Hale's method (1946), to be of the hyaluronic acid type. The material staining green appeared in the form of scattered granules which remained embedded in a matrix which took up the counter-stain (chromotrop 2R).

Alkaline phosphates in NR *bodies*

With Gomori's revised method (Glick, 1949) the presence of alkaline phosphatase was detected in the *NR* bodies. In mature forms deposition of cobalt sulphide was detected in the form of globules embedded in a dark grey matrix. In younger or budding forms such globules were scanty, but the matrix always stained grey.

DISCUSSION

NR bodies, first recorded by Hertig (1936), were said to be living agents or the manifestations of these, but details about their structure and nature were not known. Studies so far done by the authors indicate that *NR* bodies are parasitic on spermatogonial cells. The mature *NR* bodies contain both RNA and DNA. By budding they give rise to small spherules which appear as Feulgen-positive dots. The latter migrate towards the fresh spermatogonial cells and infect them. The infection brings about degeneration of the nucleus of the spermatogonial cells, resulting in the formation of a 'pathological cell' which is ultimately converted into a mature *NR* body.

It is still premature to evaluate the role which *NR* bodies play in the biology of the insects in question. It remains to be seen whether they can, by their destructive activity on reproductive cells, completely prevent the production of sperms. Can these parasites be utilized to keep down the bug population? The study of the mode of transmission of these parasites from one bug to the other calls for investigation. The probability of infecting other insects with *NR* bodies of this bug will also have to be undertaken to ascertain the specificity of this organism. It may be mentioned here that Hertwig reported negative results of his inoculation and cultivation experiments with *NR* bodies.

Hertig wondered whether the *NR* bodies of *Culex pipiens* were related to the inclusion bodies of psittacosis virus described by Bedson (1933) and Bedson & Bland (1932, 1934). But in our present state of knowledge we cannot assign these bodies to any known group of organisms. Presumably they belong to a hitherto unexplored group of organisms whose proper place is perhaps somewhere in the great chain of parasitic organisms ranging from the smallest bacterium to the largest virus.

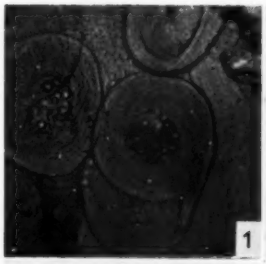
The relationship between *NR* bodies in this bug and those in *Culex pipiens* (and also those probably present in other insects), if worked out, will no doubt provide interesting information about the probable origin of these bodies.

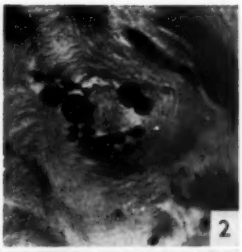
SUMMARY

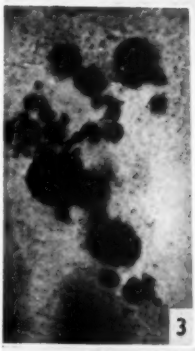
1. *NR* bodies show special affinity for neutral red. They are rounded or irregular in outline, measuring 0·25–12·5μ in diameter.

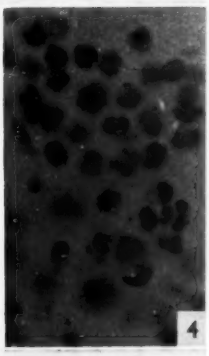
2. In fixed preparations *NR* bodies appear as spherical and vacuolated structures.

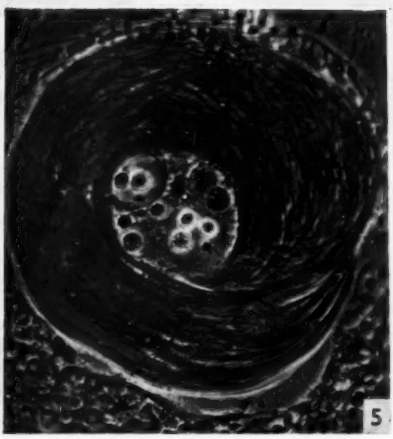
3. RNA is present in abundance in mature *NR* bodies, but while DNA is present in variable amount, the Feulgen technique shows that in their very young stage *NR* bodies are rich in DNA.











(*Facing p.* 424)

4. Glycogen is absent from the *NR* bodies, but Hale's technique provides evidence that the hyaluronic acid type of polysaccharide is present in them.

5. By Gomori's modified technique the presence of alkaline phosphatase in the *NR* body was demonstrated.

6. The mature *NR* bodies multiply by a process of budding. Small buds or spherules rich in DNA infect fresh spermatogonial cells, the latter being converted into the 'pathological cells' of Hertig.

We wish to record our thanks to Dr S. Datta, Director, Indian Veterinary Research Institute, Mukteswar, and Dr R. N. Chaudhuri, Director, School of Tropical Medicine, Calcutta, for kindly providing facilities for the work; and to the Indian Council of Agricultural Research, for awarding a Research Fellowship to one of us (B. D.). We are indebted to the authorities of the British Museum and the Zoological Survey of India for the identification of the bug.

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EXPLANATION OF PLATE VI

Photomicrographs of *NR* bodies from the testis of bed bug.

Fig. 1. Sperm-packs enclosing a group of mature *NR* bodies after staining with neutral red. × 85.
Fig. 2. Section of testis showing *NR* bodies in the process of budding. Stained with iron-haematoxylin. × 340.
Fig. 3. Same as above. × 670.
Fig. 4. Section of testis showing Feulgen reaction in the *NR* bodies in the lower part of the picture. Normally developing spermatogonial cells lie above. × 340.
Fig. 5. Phase-contrast appearance of *NR* bodies enclosed within a sperm-pack. Normal saline preparation. × 835.

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ON A NEW LUNGWORM, *PNEUMOSTRONGYLUS OVIS* SARWAR, 1953, AND SOME REMARKS ON *VARESTRONGYLUS* SPECIES FROM SHEEP AND GOATS IN PAKISTAN

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(With 3 Figures in the Text)

The genus *Pneumostrongylus* was erected by Mönnig (1932) to accommodate the species, *P. calcaratus*, recovered from the lungs of the common impalla (*Aepyceros m. melampus*) from the Transvaal. Another species, based on the tail piece of a single male lungworm, was described by Dougherty (1945), from the common white-tailed deer, *Odocoileus v. virginianus*, from North America. The present species, *P. ovis*, is based on tail pieces of three male specimens which were recovered from bronchioles of sheep and goats at Murree (7000 ft. above sea-level).

Family: Metastrongylidae Leiper, 1909

Subfamily: Protostrongylinae Kamenskii, 1905

Genus **Pneumostrongylus** Mönnig, 1932

Pneumostrongylus ovis Sarwar, 1953

The worms are filiform and extremely delicate. They appeared yellow when they were fresh.

The longest piece of the worm measures 19 mm. and has a width of 0·11 mm. in front of the bursa.

The bursa is comparatively small and consists of two lateral lobes and an indistinct dorsal lobe. The ventral rays have a common stem, which is about twice as long as the rays themselves. The antero-lateral ray is divergent from the other lateral rays; the medio- and postero-lateral rays are equal in length and are fused, except near their tips. The externo-dorsal rays arise independently and show characteristic bends in their course. The dorsal ray has a long stem, which ends distally in three digitations.

The spicules are equal and similar, and measure 0·28–0·30 mm. long. They are, however, pigmented at the commencement of their distal halves. The transversely striated alae commence at a short distance from the proximal end of the spicules and are conspicuous in their posterior portions. The gubernaculum is here described in the terminology given by Schulz, Orlow & Kutass (1933). The gubernaculum measures about 0·12 mm. long and consists of weakly sclerotized corpus and well-sclerotized crura with two stocky pedes at their distal ends. The telamon comprises two small chitinous pieces each measuring 0·035 mm. long.

Host: *Ovis aries* and *Capra hircus*.

Locality: Lungs, bronchioles.

Location: Murree (Punjab), Pakistan.

This species is differentiated from the other two species of the genus by the size of the spicules which are 0·384–0·421 mm. long in *P. calcaratus* and 0·195 mm. long in *P. tenuis*. The shape of the gubernaculum in each of the species is highly

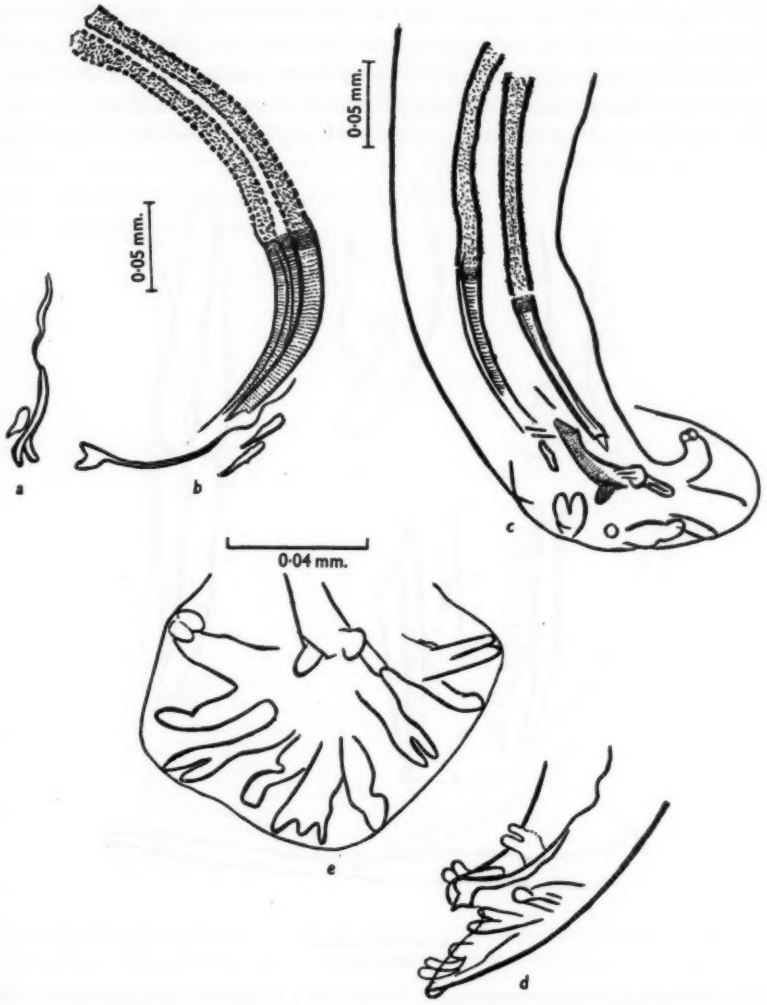

Fig. 1. *Pneumostrongylus ovis*. *a*, Gubernacular complex and telamon; *b*, lateral aspects of the spicules and gubernacular complex; *c*, *d*, dorso-ventral and lateral aspects, respectively, of the hind end of the worm; *e*, bursa.

characteristic. *P. ovis* is also differentiated from *P. calcaratus* by the limited pigmentation of its spicules, the pigmentation being confined only to a small portion of the spicules, and from *P. tenuis* by the presence in the latter of a longitudinal slit in the middle of the spicules.

The genera *Pneumostrongylus* and *Varestrongylus* have a rather close resemblance. Dougherty (1945) stated that the genus *Pneumostrongylus* showed certain resemblances to the genus *Varestrongylus* and this led him to suspect that the former might be a synonym of the latter; but later he concluded, with the help of Dr G. Dikman, that it is best to regard the two genera as being distinct. The genera *Pneumostrongylus* and *Varestrongylus*, although they are closely allied, are, however, on critical comparison, found to offer distinct generic characters. The dorsal ray in all the three species of the genus *Pneumostrongylus* ends distally in three papillae, while in species of the genus *Varestrongylus the* dorsal ray has five or six

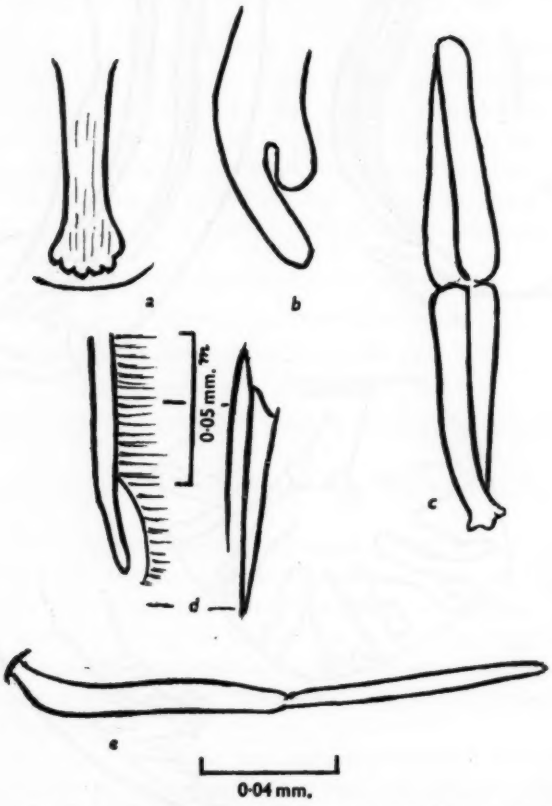

Fig. 2. *Varestrongylus pneumonicus*. *a*, Dorsal ray; *b*, medio- and postero-lateral rays; *c*, gubernaculum, dorso-ventral aspect; *d*, posterior endings of spicules; *e*, gubernaculum, lateral aspect.

papillae at its end. The postero-lateral ray in species of the genus *Varestrongylus* is very small and arises as a branch of the medio-lateral ray, while in species of the genus *Pneumostrongylus* the postero-lateral and medio-lateral rays are equal in length. The spicules in species of the genus *Pneumostrongylus* show a change in configuration in the first part of the distal half, characterized by the pigmentation in *P. calcaratus* and *P. tenuis* and a longitudinal slit in *P. tenuis*.

Because there is confusion in the terminology of the gubernacular complex, an emended definition of the genus *Pneumostrongylus* seems to be desirable.

Genus **Pneumostrongylus** Mönnig, 1932

Protostrongylinae: bursa with ventral rays close together; antero-lateral ray divergent from the fused medio- and postero-laterals, the latter separating only at their tips and being of equal length; dorsal ray ending distally in three digitations. Spicules equal and provided with transversely striated alae and pigmentation in the first portion of their distal halves. The gubernacular complex consists of a weakly sclerotized corpus and well-sclerotized crura with pedes at the distal ends. Telamon present. Oviparous, eggs segmenting when laid.

Host: Parasites of ruminants.

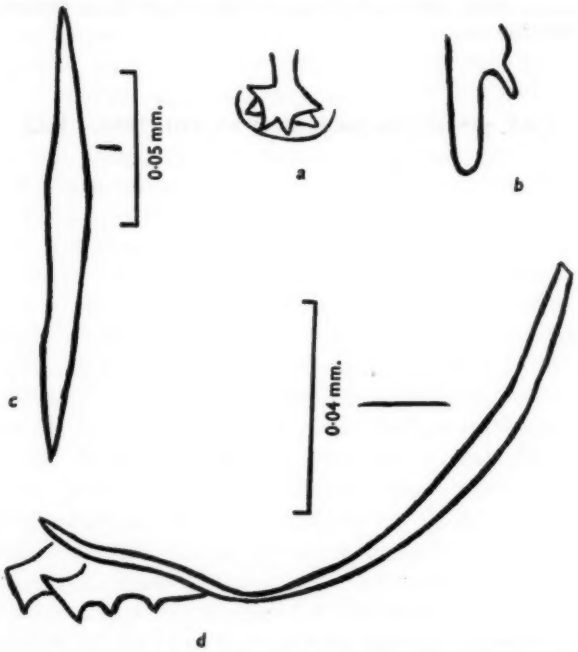

Fig. 3. *Varestrongylus capricola*. *a*, Dorsal ray; *b*, medio- and postero-lateral rays; *c*, gubernaculum, dorso-ventral aspect; *d*, gubernaculum and telamon, lateral aspect.

Varestrongylus pneumonicus Bhalerao, 1932 and *Varestrongylus capricola* Sarwar, 1944

Bhalerao (1932) states that the spicules of *V. pneumonicus* measure 0·29–0·57 mm. long, but the spicules measured by the author in seventy-five specimens of the species were found to range in length from 0·42 to 0·45 mm. The cause of this surprising disparity between Bhalerao's and the author's measurements was explained by examination of Bhalerao's type specimens deposited in the Indian Institute of Veterinary Research, Izatnagar, India. It was found that a male specimen of

V. capricola with the spicules measuring about 0·28 mm. had been mixed up with specimens of *V. pneumonicus*. The gubernaculum of *V. capricola* is reproduced (Fig. 3) for comparison with that of *V. pneumonicus* (Fig. 2).

V. pneumonicus is a very common parasite of sheep and goats throughout the Himalayas, while *V. capricola* occurs as a rare species side by side with *V. pneumonicus*.

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ON THE FAMILY PARASUBULURIDAE VAN DEN BERGHE & VUYLSTEKE, 1938, AND THE SUBFAMILY NUMIDICINAE LÓPEZ-NEYRA, 1945 (NEMATODA)

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(With 9 Figures in the Text)

Heterakis crassispiculum was described by Sonsino (1889) from a *Vulpes* species, and von Linstow (1899) described a species, *Oxynema rectum*, making it the type of a new genus, *Oxynema*. These names were shown to be synonyms (Seurat, 1915), and since then the species does not appear to have been recorded again, although two other species have since been added to the genus *Oxynema*. Of these, *Heterakis boueti* Gendre, 1911 was placed in this genus by Railliet & Henry (1914) and later a new species, *O. typicum*, was described by Kreis (1940). Baylis (1928) redescribed *O. boueti* and, finding that it possesses two spicules, placed it in the genus *Numidica* Barreto, 1919. Although the genus *Oxynema* was originally described as possessing two spicules, it was obvious from the description and the figures of *O. crassispiculum* that what had been considered to be a second small spicule was in fact a gubernaculum. Thus the genus *Oxynema* was considered to possess one spicule, while the genus *Numidica* had two. In 1930 Baylis said, 'Should this [second spicule] be present [in *Oxynema crassispiculum*], the supposed generic distinction between *Oxynema* and *Numidica* would probably break down'.

The genus *Numidica* was erected by Barreto (1919), with *Allodapa numidica* Seurat, 1915, as the type species. Since then a further three species, *Numidica monodi* Baylis, 1930, *N. suricattae* Mönnig, 1931, and *N. alata* Mazhar, 1933, have been added to it. From *Petrodromus nigriseta*, Baylis (1934) described a new species, *Numidica petrodromi*, which he considered to belong, provisionally, to this genus, but which was later removed by López-Neyra (1945) and placed, as type species, in a new genus, *Baylisnumidica*, which he erected to receive it.

In the same paper López-Neyra erected a new subfamily, Numidicinae (Subuluridae),* into which he put these three genera, namely, *Oxynema*, *Numidica* and *Baylisnumidica*.

In 1938 van den Berghe & Vuylsteke erected a new family, Parasubuluridae, and a new genus, *Parasubulura*, to take a new species they described, *P. gerardi*. This family possessed characters intermediate between those of the families Heterakidae (Railliet & Henry, 1912), and Subuluridae (Trevassos, 1914). The type species had a bucal cavity with three teeth at the base, as species of the family Subuluridae have, and a circular precloacal sucker, like that of species of the family Heterakidae. These authors further considered it probable that *Baylisnumidica petrodromi* belonged to this genus, but, because Baylis had described this

* This subfamily, given by López-Neyra as Numidinae, is here emended to Numidicinae.

species as possessing a 'preanal sucker...oval and surrounded by a border which appears to be of a muscular nature', they could not definitely decide this point.

It has been explained above that the distinction between the genera *Oxynema* and *Numidica* depends upon whether *Oxynema crassispiculum* has one spicule or two. A re-examination of this species is therefore important. The redescription given below is based on an examination of von Linstow's type material from the Zoological Museum, Berlin.

Whether or not *Baylisnumidica petrodromi* belongs to the family Subuluridae or to the family Parasubuluridae is dependent upon the shape of the precloacal sucker, and the following redescription of this species is based on a study of the type material which is in the collections of the British Museum (Natural History).

Oxynema crassispiculum (Sonsino, 1889), Seurat, 1915

Syn. *Heterakis crassispiculum* Sonsino, 1889. *Atti Soc. Tosc. Sci. Nat., Pisa*, **6**, 227.
Oxynema rectum von Linstow, 1899. *Mitt. Zool. Mus., Berl.*, **1**, 19.

Hosts: *Vulpes* sp. Sonsino (1889); *Canis megalotis* von Linstow (1899); *C. vulpecula* von Linstow (1899); *Vulpes bengalensis*, present author, from a collection made by the late Dr W. N. F. Woodland in India.

Position: Vermiform appendix.

Locality: Egypt; India.

Three male and three female specimens were available from von Linstow's material. The worms are fairly short, the males being 8–12 mm. in total length and 0·32 mm. wide, while the females are 12–16 mm. long, and they are stout, measuring 0·4 mm. at the widest part of the body. The body tapers from about the level of the oesophageal bulb to a fairly small head, 0·088 mm. in diameter. The mouth is bounded by two indistinct lips, being elongate in the dorso-ventral direction, with six small papillae surrounding it. It opens into a distinct buccal capsule, which is divided into two parts, a narrow portion 0·05 mm. long by 0·04 mm. wide anteriorly and a globular portion 0·06 mm. wide by 0·03 mm. in depth posteriorly. The posterior portion has three large teeth at the base. The buccal capsule is followed by a small, indistinct, muscular pharynx 0·05 mm. in length (Fig. 1), which connects the buccal capsule with the oesophagus proper.

The oesophagus extends backwards for 1·29–1·92 mm. in the male and 1·87–2·10 mm. in the female, expanding slightly at the posterior end until it is 0·12–0·17 mm. in width. It then contracts sharply to join the posterior oesophageal bulb by a narrow isthmus. The bulb is 0·21–0·34 mm. in length and 0·22–0·36 mm. in width. The nerve ring encircles the oesophagus 0·15–0·28 mm. from the anterior end, and the excretory pore, which opens on the ventral surface from an excretory sac, lies 0·42–0·61 mm. from the head. There are no lateral alae. The body has fine cuticular striations 18–26μ apart.

The male tail, which is tightly curled ventrally and has a fine terminal spike, is 0·23–0·31 mm. in total length, including the terminal spike, which is about 0·05 mm. in length. The posterior lip of the cloacal opening bears a cushion-like swelling covered by fine granulations. Lying 0·3 mm. in front of the cloacal opening is the

elongate pre-anal sucker, which is 0·091–0·099 mm. in length and is bounded by longitudinal ridges developed from the cuticle. The sucker is flanked by a pair of sessile papillae. There are a further nine or ten pairs of sessile papillae, one pair lying approximately half-way between the pre-anal sucker and the cloacal opening, and two pairs which vary in position and may be either pre-cloacal, cloacal or post-cloacal in position (Figs. 3, 4). Von Linstow figures these papillae as being post-cloacal. There are also five or six pairs of post-cloacal papillae, one lying immediately behind and sublateral to the swollen posterior lip of the cloacal opening, two or three of them grouped just anterior to the commencement of the terminal spike and one pair lying between this group and the immediately post-cloacal pair. The last of the post-cloacal pairs lies lateral to the terminal group of two or three (Figs. 2–4).

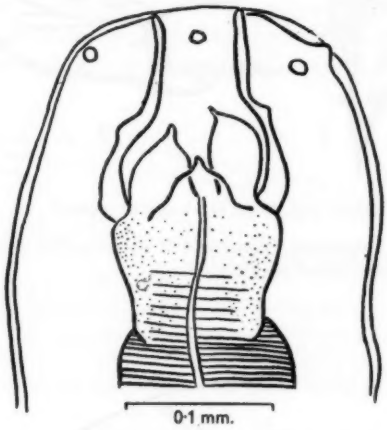

Fig. 1. *O. crassispiculum* (Sonsino). View of the head from the dorsal surface.

There are two spicules of vastly different shape and size. The larger, right, spicule is strongly alate and 0·029–0·051 mm. in width and 0·58–0·71 mm. in length. The smaller, left, spicule is alate and very poorly 'chitinized'. It is extremely difficult to see, because, in a lateral view it is masked by the bulk of the right spicule and in a ventral view its poor chitinization renders it almost transparent, so that its exact limits are difficult to determine with accuracy. It is about 0·47–0·55 mm. in length and 9μ in width at the distal end (Fig. 4). The gubernaculum is fairly heavily built, being 0·15–0·18 mm. in length.

In the female the vulva occurs 5·5–7·5 mm. from the anterior end of the body and is bounded by prominent lips. The uteri are opposed and in the mature individuals they run forward to within 0·15 mm. of the oesophageal bulb and backwards to the level of the anus. The eggs are subglobular at first, but, when they are fully embryonated, as they are *in utero*, they are oval, 76–78μ long and 46–56μ wide. They are thin-shelled and contain fully developed, vermiform embryos.

The tail in the female is long with a fine terminal spike, its total length, including the spike, being 1·92 mm.

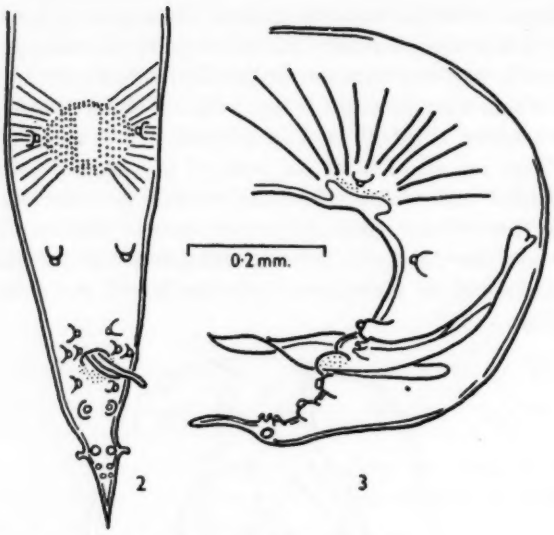

Fig. 2. *O. crassispiculum*. Male tail, from the ventral aspect.

Fig. 3. *O. crassispiculum*. Male tail, from the left showing the structure of the right spicule and one arrangement of the caudal papillae.

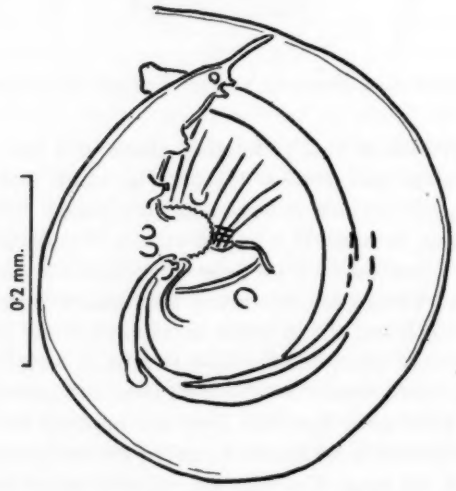

Fig. 4. *O. crassispiculum*. Male tail, from the left showing the left spicule and a second arrangement of the caudal papillae.

Parasubulura petrodromi (Baylis, 1934), nov.comb.

Syn. *Numidica petrodromi* Baylis, 1934. *Ann. Mag. Nat. Hist.* Ser. 10, **13**, 338.
Baylisnumidica petrodromi López-Neyra, 1945. *Rev. ibér. Parasit.* **5**, 271.

Host: *Petrodromus nigriseta.*

Position: Caecum.

Locality: Tanganyika Territory.

The male is 11 mm. in length and 0·5 mm. in breadth. Immediately behind the head, which is 0·06–0·07 mm. in diameter, the neck thickens rapidly, the cuticle being particularly thick at this point. The buccal capsule, 0·026–0·028 mm. in depth, is simple and is not divided into two distinct parts. It has definite, thick, cuticular walls. At the anterior end of the pharynx, which is 0·09 mm. in length, are located three blunt, tooth-like structures, which are developments from the cuticular lining of the pharynx (Fig. 6). These structures are identical with those figured by van den Berghe & Vuylsteke (1938) as occurring in *Petrodromus gerardi.*

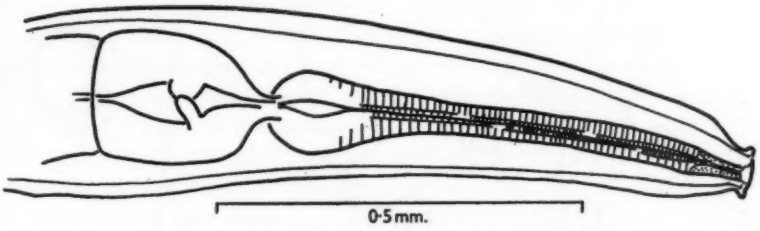

Fig. 5. *P. petrodromi.* Lateral view of the anterior end of the body.

The oesophagus runs back 1·1 mm., expanding until it is 0·11 mm. in width, and it then contracts sharply to join the posterior oesophageal bulb by a narrow isthmus (Fig. 5). The bulb is 0·27 mm. wide and 0·29 mm. long. The nerve ring encircles the oesophagus 0·24–0·27 mm. from the head and the excretory pore opens on the ventral surface 0·2 mm. behind it. The body is covered by fine, transverse striations and has no cervical alae.

The tail, which is 0·15 mm. long, tapers to a fine point and bears a circular precloacal sucker and ten pairs of sessile papillae. The cloacal sucker is 0·15 mm. in external diameter and lies 0·65–0·69 mm. anterior to the cloacal opening. Its lining, which is protruding in all the specimens present, is covered by a regular series of cuticular bosses. The rim is composed of an inner continuous ring of cuticle with an external ring made up of a series of about sixty or seventy radiating lamellae, at the base of each of which there is a small papilla (Fig. 9). The caudal papillae are arranged as in Figs. 8 and 9. One pair (Fig. 9*a*) flanks the precloacal sucker (*s.*), one pair (Fig. 9*b*) lies between the sucker and the cloacal opening (*cl.*), two pairs (Figs. 8 and 9*c*, *d*) are cloacal, and two pairs (Fig. 8, *e*, *f*) lie outside these. There are four pairs of post cloacal papillae (Figs. 8 and 9, *g*, *h*, *i*, *j*); the terminal group of two pairs (*i*, *j*) being much smaller than the others.

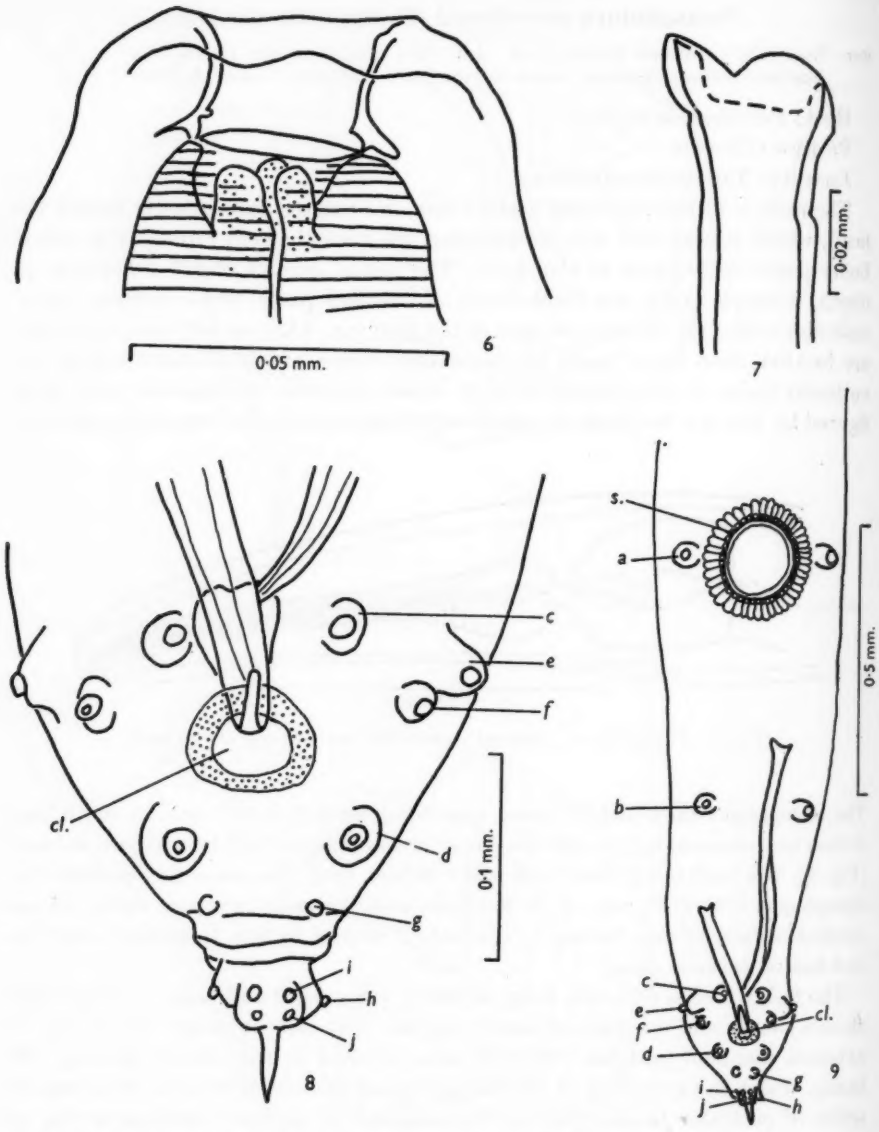

Fig. 6. *P. petrodromi*. Detailed view of the anterior end of the body from the lateral aspect.

Fig. 7. *P. petrodromi*. Anterior end of the left spicule from the left side.

Fig. 8. *P. petrodromi*. Ventral view of the posterior end of the male tail, showing the terminal papillae in detail.

Fig. 9. *P. petrodromi*. Ventral view of the male tail, showing the structure of the pre-cloacal sucker (semi-diagrammatic) and the arrangement of the caudal papillae.

The spicules are subequal. The right is 0·46–0·5 mm. and the left 0·43–0·47 mm. in length. The two spicules are identical in structure, both being slightly alate for the proximal two-thirds of their length. Their distal ends are slightly cup-shaped (Fig. 7). The gubernaculum is 0·13–0·15 mm. in length. The posterior lip of the cloacal opening is cushioned and covered by very fine cuticular granulations.

The female is 14–15 mm. in total length and 0·55–0·65 mm. in breadth at the vulva. The oesophagus is 0·55–0·66 mm. in total length. The tail, which tapers to a fine point, is 0·42–0·45 mm. in length. The vulva is situated at about the middle of the body. The eggs are round, 70–90 μ in diameter and embryonate *in utero*.

SYSTEMATIC DISCUSSION

It has been explained above that the differences between the two genera *Numidica* and *Oxynema* are very slight and that these two genera are separated mainly by the presence of two spicules or only one. The present examination of *O. crassispiculum* shows that it possesses two spicules and is in all other ways similar to the species placed in the genus *Numidica*. The posterior lip of the cloacal opening of the male is raised into a cushion-like swelling bearing small granulations. The caudal papillae are pedunculate and the pre-cloacal sucker is of the same shape and form. Thus the distinction between the two genera *Numidica* and *Oxynema* disappears and *Numidica* becomes a synonym of *Oxynema*.

The examination of *Baylisnumidica petrodromi* shows that the structure of the head and the pre-cloacal sucker make it necessary to remove this species to the genus *Parasubulura* (see below). Thus the subfamily Numidicinae now contains only one genus, *Oxynema*, of which the generic diagnosis is given below:

Subfamily NUMIDICINAE López-Neyra, 1945

Syn. Numidinae López-Neyra, 1945, lapsus calami.

Genus OXYNEMA von Linstow, 1899

Syn. *Numidica* Barreto, 1919, nov.syn.

Subuluridae; Numidicinae. Two indistinct lips with six cephalic papillae. Buccal capsule divided into two parts, an anterior and a posterior, the latter with three teeth at its base. Lateral cervical alae absent. Male with very dissimilar spicules; the right heavily cuticularized with folded alae, the left very poorly so, alate and indistinct. A gubernaculum present. About ten pairs of pedunculate papillae. Pre-anal sucker elliptical, bordered by parallel cuticular prominences. Posterior lip of the cloacal opening in the male raised into a cushion-like swelling bearing cuticular granulations. Eggs subglobular, fully embryonate when laid.

The genus *Oxynema* now contains the following six species:

Oxynema crassispiculum (Sonsino, 1889)

Syn. Given above.

Oxynema boueti (Gendre, 1911)

Syn. *Heterakis boueti* Gendre, 1911. *Proc. Soc. Linn. Bordeaux*, **65**, 68.
Subulura boueti Travassos, 1913. *Tese apresentada Fac. Med. Rio de Janeiro.*
Oxynema boueti Railliet & Henry, 1914. *Ext. IXe Congres Int. Zool., Monaco*, p. 674.
Numidica boueti Baylis, 1930. *Bull. Mus. Hist. nat. Paris*, **2**, 117.

Host: *Xerus erythropus.*
Position: Intestine.
Locality: Africa.

Oxynema numidica (Seurat, 1915), nov.comb.

Syn. *Allodopa numidica* Seurat, 1915. *C.R. Soc. Biol., Paris*, **78**, 122.
Numidica numidica Barreto, 1919. *Mem. Inst. Osw. Cruz*, **11**, 57.

Host: *Vulpes vulpes.*
Position: Intestine.
Locality: Africa.

Oxynema monodi (Baylis, 1930), nov.comb.

Syn. *Numidica monodi* Baylis, 1930. *Bull. Mus. Hist. nat. Paris*, **2**, 117.
Numidica menodi Skrjavin, 1949, lapsus [*Parasitic Nematodes and the Illnesses Caused by Them*, 1], Moscow.

Host: *Numida meleagris galeata.**
Position: Caecum.
Locality: Near Bamako on the Niger, Africa.

Oxynema suricattae (Mönnig, 1931), nov.comb.

Syn. *Numidica suricattae* Mönnig, 1931. *Rep. Director Vet. Serv. Anim. Indust., Union of South Africa*, **17**, 277.

Host: *Suricatta suricatta.*
Position: Large and small intestines.
Locality: Dealesville, Orange Free State.

Oxynema alata (Mazhar, 1933), nov.comb.

Syn. *Numidica alata* Mazhar, 1933. *Z. Parasitenk.* **6** (2), 269.

Host: *Vulpes (V) vulpes.*
Position: Intestine.
Locality: Aligarh, India.

The species described above are all very similar and are separated mainly on the basis of the different lengths of their body-parts. Although several of them are probably synonymous, it will require a re-examination of the type specimens before this can be definitely decided.

Kreis (1940) described as new a species obtained from a 'Nager' which he placed in the genus *Oxynema*. This species, *O. typicum*, he describes as having only one spicule, but it appears to fit the genus *Oxynema*, as redefined above, in all other

* The host is given in the original description as *Numida* sp., but Baylis has written the name given above in the margin of his personal copy of his paper, which I have seen.

respects. However, until the type specimens have been re-examined, it is impossible to place it definitely within the genus *Oxynema* and it must, for the time being, be regarded as a species *incertae sedis*.

Subfamily PARASUBULURINAE (Berghe & Vuylsteke, 1938), nov.s.fam.

Subuluridae Yorke & Maplestone, 1914. Small or medium-sized worms without well-defined lips. Distinct buccal capsule. Indistinct pharynx with three tooth-like structures developed from the cuticular lining of the anterior portion of the pharynx. Oesophagus with a posterior bulb. Pre-cloacal sucker of the male circular and bounded by a series of radiating cuticular lamellae.

It has been explained above that the family Parasubuluridae was considered by Berghe & Vuylsteke (1938) to be intermediate between the families Heterakidae and Subuluridae. However, the pre-cloacal sucker, although it is circular in shape, is not heterakid in structure. It does not possess the definite rim which is so characteristic of the Heterakidae but suggests, rather, an elaboration of the sucker-type found in the Subuluridae, Numidicinae.

Further, the head is of the same type as that found in the Subuluridae, possessing the distinct buccal capsule with three tooth-like structures at the base which are typical of the family. These characters are as consistent in their presence as is the elongate sucker.

The families Heterakidae and Subuluridae can be separated by the structure of the anterior end as well as by the shape of the pre-cloacal sucker. The characters of the anterior end are probably more indicative of the relationships between these two families than is the structure and shape of the pre-cloacal sucker. The erection of the family Parasubuluridae by Berghe & Vuylsteke is the result of an over-simplification of the relationships between the families Heterakidae and Subuluridae, in which they have placed too much importance on the shape of the pre-cloacal sucker and too little on the details of the anterior end of the body. The family Parasubuluridae is, therefore, reduced in rank and considered a subfamily of the family Subuluridae.

Skrjabin & Schikhobalova (1948) erected the genus *Heterobulura* for a species described by Sandground (1933) and named by him *Subulura callosa*. This genus is considered to be distinct from the genus *Parasubulura* in that Sandground described it as possessing pedunculate papillae.

The subfamily Parasubulurinae contains the following two genera and three species.

Genus **Parasubulura** Berghe & Vuylsteke, 1938

Syn. *Baylisnumidica* López-Neyra, 1945, nov.syn.

Parasubulum gerardi Berghe & Vuylsteke, 1938

Host: *Petrodromus tetradactylus*.
Position: Intestine.
Locality: Belgian Congo.

Parasubulura petrodromi (Baylis, 1934), nov.comb.

Syn. Given above.

Genus **Heterobulura** Skrjabin & Schikhobalova, 1948

Heterobulura callosa (Sandground, 1933) Skrjabin & Schikhobalova, 1948

Mentioned by Skrjabin 1949 in [*Parasitic Nematodes and the Illnesses Caused by them*], Moscow.

Syn. *Subulura callosa* Sandground, 1933. *Bull. Mus. Comp. Zool. Harv.* **75** (6), 276.

Host: *Petrodromus tetradactylus*.

Position: Large intestine.

Locality: Mount Silinda, Southern Rhodesia.

SUMMARY

1. *Oxynema crassispiculum* (Sonsino, 1889) has been redescribed and shown to possess two spicules.

2. The genus *Numidica* Barreto, 1919 has been shown to be synonymous with the genus *Oxynema* von Linstow, 1899.

3. The genus *Oxynema* has been redefined and a synonymic list is given of the species that it now contains.

4. *Oxynema typicum* Kreis, 1940 is considered to be a species *incertae sedis*.

5. *Baylisnumidica petrodromi* (Baylis, 1934) has been redescribed and shown to belong to the genus *Parasubulura* Berghe & Vuylsteke, 1938.

6. The family Parasubuluridae is reduced to subfamily rank, and placed in the family Subuluridae.

The author wishes to express his thanks to Dr G. Hartwich of the Zoological Museum, Berlin, for allowing him to examine von Linstow's type material, and also to Dr H. W. Parker, Dr G. O. Evans and Mr S. Prudhoe, of the British Museum (Natural History), for help given during the writing of this paper.

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THE BACTERIA ASSOCIATED WITH *TRIATOMA INFESTANS* AND SOME OTHER SPECIES OF REDUVIIDAE

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INTRODUCTION

The existence of bacteria in the intestine of the blood-sucking Reduviid *Rhodnius prolixus* has been firmly established by the work of Wigglesworth (1936) and Brecher & Wigglesworth (1944). Before these accounts were published, little was known about the symbiotic micro-organisms of carnivorous Hemiptera, apart from a brief report by Duncan (1926), and no mention is to be found in the great work of Buchner (1930). By contrast, the bacteria associated with the phytophagous members of this Order have been known for many years. Although predatory insects have not usually been found to contain bacterial associates, the discovery of such symbionts in *R. prolixus* was not unexpected, because other insects that feed solely on blood throughout life, the louse and tse-tse fly, are known to harbour bacteria. It has been supposed that the function of these micro-organisms is to synthesize accessory nutritional factors which are lacking in the blood diet, and this has been confirmed by Brecher & Wigglesworth (1944), who failed to obtain growth beyond the fifth instar with nymphs reared in a sterile manner. Other species of blood-sucking Reduviidae have also been found to contain bacteria in their intestines, but, while the symbiont of *R. prolixus* has been identified and described as *Actinomyces rhodnii* Erikson, 1935, the nature of the symbionts found in *Triatoma* spp. is still obscure. Brecher & Wigglesworth (1944) have recorded *A. rhodnii* and *Streptococcus faecalis* from *Triatoma infestans*, and Wigglesworth (1936) found organisms similar to those of *R. prolixus*, in *T. rubrofasciata*, *T. infestans*, and *Eutriatoma flavida*. Organisms of the *Rickettsia* type have been recorded from *T. rubrofasciata* by Webb (1940). There do not appear to be any records of bacterial isolations from any of the predatory species of Reduviidae. In the work here recorded, the bacterial flora of *T. infestans* has been studied, not only because it is little known, but also from the point of view of a comparison between the predatory and the blood-sucking Reduviidae. It seemed likely that *Triatoma* might provide a useful contribution to such a study, because there are good reasons for regarding this genus as being less highly adapted to the blood-sucking habit than *Rhodnius* (Goodchild, 1955; also Baptist, 1941, for salivary gland structure). In addition, the author has examined the available material of predatory types of Reduviidae for the presence of bacteria. These investigations have been limited to the intestines of the insects concerned, because no extra-intestinal mycetomes were found in the species examined.

TECHNIQUE

Three kinds of experimental technique were used for revealing the presence of micro-organisms within the insects. These were: (1) stained smears of mid-gut contents and tissues; (2) serial sections, 5μ thick, of mid-gut tissue fixed with Bouin's fluid; (3) inoculations on to slopes of blood-agar medium. The stains used were Gram's or Wayson's for smears, and Gram-Twort for sections. Inoculum for the bacterial cultures was prepared in the following manner. The insects were first sterilized by immersion in 0·1 % aqueous mercuric chloride for 5 min. They were then washed with at least three changes of sterile water and put into a sterile Petri dish lined with filter-paper, where they remained until they were dry. Each in turn was then dissected in sterile saline and the tissue to be cultured was removed to a sterile microscope slide and broken up with the flat ground end of a sterile glass rod. A drop of bacteriological broth was added, and the material was inoculated in the usual way. In addition to *Triatoma* spp., material of *Rhodnius prolixus* was used in each of these three techniques, as an indicator of their efficiency in revealing the presence of micro-organisms.

It was found that the third method gave the most reliable results, abundant growth appearing on the inoculated slopes, but little or none on control slopes inoculated with saline or broth which had been exposed to the air under the same conditions. Serial sections of mid-gut tissue were also satisfactory, but the best results were obtained when the specimens of *R. prolixus* had been recently fed. Stained smears of mid-gut tissue were usually satisfactory with *T. infestans*, but they gave very poor results with *R. prolixus*. Because this technique offered the quickest method for the examination of large numbers of insects, the reason for its failure with *R. prolixus* was examined more closely. There was no doubt of the presence of *Actinomyces rhodnii*, because the bacterial isolations of this organism were all successful. The author therefore decided to deal with this problem by making inoculations on to hanging drops of agar, so that the drops could be examined microscopically at all stages. This procedure revealed that the cultures originated in small coccus-like forms, which in smears and sections do not stain very conspicuously, and are distinguished with difficulty from the granular protoplasm of the cells. The filamentous forms of *A. rhodnii*, which are more easily seen in stained preparations, are found only in the early stages of a culture, and later break up into strings of cocci. They may be found in masses in the lumen of the mid-gut of insects which have fed up to 3 or 4 days previously, but cannot be detected inside the cells. It seemed, therefore, that the tissue smear technique could not be relied upon to reveal micro-organisms, even though they were known to be present; and for this reason it was used only in conjunction with the slope-culture technique.

THE BACTERIAL FLORA OF *TRIATOMA INFESTANS*

The presence of bacteria in the mid-gut of this species was clearly demonstrated in tissue smears, in which abundant Gram-positive, rod-like organisms could be seen. In sectioned material, these were found to be enclosed within large vacuoles

in some of the cells of the mid-gut endothelium, although no regular arrangement of these cells was apparent. The lumen of the intestine, on the other hand, contained no conspicuous aggregations of bacteria, except in a few instances in which organisms of the *A. rhodnii* type and clumps of Gram-positive cocci were the only micro-organisms present. It is noteworthy that *A. rhodnii* is never found inside the cells of *R. prolixus*. Wigglesworth (1936) figured these organisms in this position, but later Brecher & Wigglesworth (1944) abandoned this view as a misinterpretation. While this later opinion is undoubtedly correct so far as the filamentous forms of *A. rhodnii* are concerned, it is still uncertain whether the minute coccal forms of this organism are maintained, between blood-meals, within the mid-gut cells, or whether a sufficient number of them are retained in the crevices between the bulbous cell-tips, to provide the inoculum for the vigorous growth which takes place in each new blood-meal.

The results obtained with the slope-cultures of *T. infestans* were rather variable, although there was a considerable uniformity among insects reared in the same cage, a fact which shows that some cross-infection must occur. It has been assumed by the author that the mode of transmission of the *T. infestans* bacteria is the same as that recorded for *A. rhodnii*, namely, through the coprophagous habits of the newly hatched nymphs. This habit was easily observed in the nymphs of *T. infestans*.

From one batch of eight *T. infestans*, pure cultures of *A. rhodnii* were obtained. This organism has a very mild anti-biotic effect towards some other bacteria and it always appeared in pure culture. No other species of bacteria was isolated from *R. prolixus*. In the majority of the isolations from *T. infestans*, however, a mixture of species occurred, and the bacterial culture technique was modified by spreading the inoculum over the surface of agar plates. By this means, individual colonies were obtained, which could then be subcultured as pure lines. Blood agar was used, as before, and also Lemco agar, which gave equally good growth. Using this technique, a survey was made of the bacterial flora of twenty-six unfed adult *T. infestans*. In addition to examining in detail the range of organisms inhabiting *T. infestans*, it was hoped that, as a result of taking only recently moulted and unfed bugs, some idea would be obtained of the importance of these organisms for successful moulting. Most of the insects in this experiment yielded an abundant growth of bacteria, with four types predominating. Details of these are given in Table 1. The first three named in this table formed small colonies, and it could be seen that the plate cultures derived from a rich inoculum. The typical appearance was that the first streaks on the plate formed a continuous mass of growth, the separate small colonies being found only in the last streaks to be made. The species identified as resembling *Bacterium alkaligenes* gave very spreading growth, and might have been present in the inoculum in small numbers only. It did not occur on control plates and was probably a genuine isolate from the insect's tissues, but its presence there cannot be regarded, without further evidence, as other than accidental. It will be noted that it did not occur in pure culture in any of the insects. In addition to these four abundant types of bacteria, two insects yielded only a few colonies of an organism resembling *A. rhodnii*, and two were sterile. In a subsidiary experiment, with six males which had been starved for a long

period, only two gave a satisfactory growth of bacteria. The proportion of sterile plates in the main experiment is hardly enough to suggest that the bacteria are of no value to the insect, but it is clear that more than one bacterial species can fulfil the symbiotic function in *T. infestans*. It should be mentioned that a few bacterial colonies were seen which appeared to differ from those selected for identification. It was considered that these were not sufficiently abundant to exclude completely the possibility of chance contamination, or to be of any significance in the biology of the insect.

Table 1. *Bacteria isolated from* Triatoma infestans

Type no.	No. of cultures	Morphology after 24 hr. at 37° C.	Appearance of colonies	Action on sugars* (10 days)	Action on litmus milk (10 days)	Identity (as far as examined), resembles
1	Ten (six pure)	Gram-positive rods, diphtheroid arrangement	Small, 0·25–0·5 mm., opaque, pinkish, round, entire edge, glistening surface	No action	Slightly alkaline	*Corynebacterium hofmannii*
2	Six (five pure)	Gram-positive streptomyces	Very small, up to 0·25 mm., opaque, greyish, round, entire, dull surface	No action	Alkaline	*Streptomyces* sp.
3	Five (three pure)	Gram-positive large staphylococcus or micrococcus	Colonies 1–2 mm., white, opaque, round, entire, shiny	Acid (no gas) after 24 hr., all except salicin	Acid and clot	*Staphylococcus albus* (or *Micrococcus* sp.)
4	Five (none pure)	Gram-negative motile rods in a matrix	Large, spreading to 5 mm., white, opaque centre, translucent edges, glistening	No action	Alkaline	*Bacterium alkaligenes*

* Glucose, maltose, mannitol, lactose, sucrose, and salicin.

The organism appearing most frequently in these cultures, identified as resembling *Corynebacterium hofmannii*, is almost certainly the same as that seen in the vacuoles in the mid-gut cells. Its frequent occurrence, not only in the plate cultures but, as typical pink colonies, in the original slope cultures, and its morphological resemblance to the organism seen most often in smears and sections, support this conclusion. This is not the first record of this genus of bacterium from blood-sucking Hemiptera, because Steinhaus (1946) isolated a similar organism from the bed-bug (*Cimex lectularius*) and named it *C. paurometabolum*. Bacteria of this type are not common as air-borne contaminants, and their association with blood-sucking Hemiptera probably has a biological significance.

OTHER *TRIATOMA* SPECIES

In addition to the material of *T. infestans*, which was from a colony that had been maintained in the laboratory for more than twenty years, it was possible to examine some specimens of *T. protracta* not long previously collected in California by Dr R. S. Ryckman. Ten specimens were used for bacteriological examination. With five of these, the anterior part of the mid-gut was divided into two pieces, one of which was used for the bacterial culture, and the other fixed and sectioned.

This task was easier with *T. protracta* than it would have been with *T. infestans*, because the tubular anterior region of the mid-gut is longer in the former species. Of the ten specimens, four yielded pure cultures of a *Staphylococcus* sp., and three yielded pure cultures of *Corynebacterium* spp. (of which one culture resembled that found in *T. infestans*). Fixed tissues from one insect which had yielded a *Staphylococcus* culture, and from another which had yielded a *Corynebacterium* culture, were examined. In both of these, organisms could be seen deep among the endothelial cells, the *Corynebacterium* in rounded vacuoles as seen in *T. infestans*, and the *Staphylococcus* in a more irregular mass which might be interpreted as a deep endothelial crypt. No organisms were seen in the lumen of the intestine. As far as could be judged, the organisms were of the morphological type found in the corresponding culture.

In view of the fact that these specimens of *T. protracta* had been kept in the laboratory for only a short period, and had not had time to become infected from the *T. infestans* stock, the occurrence of *Corynebacterium* spp. is of great interest, and suggests that this type of organism in *T. infestans* is part of its natural flora, retained through many generations of artificial culture.

Among the material received from California was a small batch of *Paratriatoma hirsuta*. These failed to thrive, and, when only two remained alive, they were sacrificed and bacterial cultures made from the mid-guts. It may be significant that no satisfactory bacterial growth was obtained from them.

THE *TRIATOMA* BACTERIA IN *RHODNIUS PROLIXUS*

The occurrence of *A. rhodnii* in some individuals of *T. infestans* has already been mentioned. Although *R. prolixus* is not normally found to harbour any species of micro-organism other than *A. rhodnii*, an attempt was made to inoculate this species of bug with bacterial strains isolated from *T. infestans*. Suitable batches of *R. prolixus* eggs were taken, on the pieces of filter-paper on which they were laid, and sterilized by the method previously described. In this case, however, the last traces of mercuric chloride were removed by washing with Lugol's iodine solution and then with dilute sodium thiosulphate, before the final rinse with sterile water. The eggs were put into six groups, and the following treatments applied:

Group 1. Inoculated with *Corynebacterium* sp., strain A.
Group 2. Inoculated with *Corynebacterium* sp., strain A, and with *A. rhodnii*.
Group 3. Inoculated with *Corynebacterium* sp., strain B.
Group 4. Inoculated with *Corynebacterium* sp., strain B, and with *A. rhodnii*.
Group 5. No inoculation.
Group 6. No inoculation.

The two strains of *Corynebacterium* differed in the colour of their colonies, and no attempt was made to differentiate them further. Inoculation was carried out when the sterilized eggs were transferred to the culture tubes, a loopful of a thick suspension of the micro-organisms in sterile saline being dropped on each egg cluster.

The culture tubes for the insects were $2 \times 1\frac{1}{4}$ in. specimen tubes, lined with filter-paper, and closed by a perforated cork covered with two layers of fine muslin. The half-inch hole in the cork was lightly plugged with cotton-wool, and the top of the tube enclosed within an aluminium cap. These tubes were sterilized before use. When the eggs hatched and the bugs required feeding, the cap, cork, and one layer of muslin were removed, and the remaining muslin layer was stretched over the mouth of the tube and secured with a rubber band. The tube was applied to a rabbit's ear which had been sterilized by wiping with alcohol. After feeding had taken place, a fresh sterile muslin, cork and cap, were fitted.

It was found that the insects which had received the inoculations with *A. rhodnii* grew normally, but those which had received *Corynebacterium* alone were greatly retarded, and did not show any better growth than the control groups which were not inoculated. When groups 2 and 4 (with *A. rhodnii*) contained nine adults and ten fifth-instar nymphs, the other four groups contained altogether two adults, twenty-two fifth-instar, and three fourth-instar nymphs. At this stage, sample bugs were taken and bacterial isolations were made from the mid-guts. These confirmed the presence of *A. rhodnii* in groups 2 and 4 and the absence of this organism from the other groups. The *Corynebacterium* were not recovered. They had evidently failed to establish themselves, although, during the 2-month duration of the experiment, some extraneous infection had occurred and *Staphylococcus* sp. was isolated from one of these insects. It would appear, therefore, that *R. prolixus* is not a suitable host for the *Corynebacterium* spp. found in *T. infestans*, perhaps because the bacteria are not able to enter into the close relationship with the epithelial cells of an unnatural host.

THE PREDATORY REDUVIIDAE

The predatory members of the family Reduviidae are not easy to rear in the laboratory and the available material has been much less abundant than that of blood-sucking species. Comparatively small numbers of the British species, *Coranus subapterus*, and of the American species, *Sinea diadema* have been subjected to bacteriological examination. *Coranus subapterus* was studied by the agar slope-culture and tissue-smear methods. Eight slopes of blood agar were inoculated, but no growth took place on any of them. Eleven smears of mid-gut tissue and contents were made, and in one of these there were fair numbers of a short rod-like organism, but no bacteria were seen in the others. The odd positive result might be attributed to recently ingested prey. Of the first consignment of *Sinea diadema* received (through the courtesy of the Canadian Department of Agriculture), three were tested by the slope-inoculation method, and gave good growth of rod-like and coccal bacteria, and yeasts. These particular insects, however, had been fed on non-sterile house-fly maggots, and it was thought possible that this might be the source of infection with micro-organisms. A later consignment of *S. diadema* was studied in more detail, and the insects were dealt with immediately on arrival, no food being given. It was understood that between collection and despatch, they had been fed on grass-inhabiting aphids and other small Homoptera, which are

their natural diet. The inoculations of intestinal material were made on to plates of Lemco and heart-digest agar, and with these insects the mid-gut and rectum were tested separately. Ten bugs were used and bacterial growth was obtained from three of them. In two of these, only the rectum material yielded bacteria. In the other, bacteria were obtained from both the mid-gut and the rectum, and those obtained from both situations were of the same type. They were identified as *Staphylococcus* sp., as was one of the other isolates also. The remaining culture, which was from the rectum of the insect, has been tentatively identified as a bacillus of the *coli* type. The other culture-plates were at first sterile, but, after 6 weeks' incubation at room temperature, growth of mould colonies was seen. They are thought to be aerial contaminants. Where bacterial growth appeared, it did not increase significantly after the first week. It was noticed that the growth appeared to come from an abundant inoculum, as happens also with typical *T. infestans* plates, so that, with regard to bacterial infection, *S. diadema* seemed to show an 'all-or-none' type of behaviour.

SUMMARY AND CONCLUSIONS

An account is given of the bacterial flora isolated from the blood-sucking Reduviid bugs, *Triatoma infestans* and *T. protracta*, and the predatory Reduviid, *Sinea diadema*. The micro-organisms which have been found in *Triatoma* spp. differ from that found in *Rhodnius prolixus* (*Actinomyces rhodnii*), in both specific identity and in their relation to the cells of the mid-gut. The symbiont occurring most frequently in *T. infestans* has been identified as a diphtheroid organism resembling *Corynebacterium hofmannii*, and sections show this organism inside vacuoles within some of the endothelial cells of the insect's intestine. The organism occurring in *R. prolixus*, on the other hand, cannot be found in this situation, but remains in the lumen of the gut. While the occurrence of the bacteria found in *T. infestans* in so intimate a connexion with the cells of its host seems to suggest a more highly organized state of affairs, *T. infestans* does not show the fixed association with one species of micro-organism which is shown by *R. prolixus*. Other bacteria occur commonly in *T. infestans*, although they do not occur in cell-vacuoles; and the range of bacterial types is so great that it seems unlikely that the species recorded in this paper are the only ones that may occur. The presence of these other bacteria may be due either to the anti-biotic action of the *Corynebacterium* sp. being weaker than that of *A. rhodnii*, or to masking of the anti-biotic action by the cell wall that encloses the organisms. The situation in *R. prolixus* shows clearly that a constant symbiotic association with a micro-organism can be maintained without the presence of specialized organs of the mycetome type.

With regard to the predatory species, no general conclusion can be reached on the evidence so far available. Compared with the blood-sucking Reduviids, insects of this kind usually have powerful proteolytic enzymes secreted in the anterior mid-gut, while the same region of the blood-suckers is a simple storage and concentration sac, digestion taking place lower down the intestine. Thus the region of the gut where bacteria are found in blood-suckers might not be a favourable

situation for them in predatory bugs. Hence it is perhaps significant that most of the few successful isolations from a predatory type were from the rectum of the insect. It is the usual opinion that insects with a predatory diet do not need bacterial symbionts, but the aim in examining the predatory Reduviidae has been to find out whether they show a condition which might have evolved into that found in their blood-sucking relatives. The search is still in progress for a rather unspecialized type of predatory Reduviid in which the salivary and anterior mid-gut digestion is less strongly developed than in those species which have been available for study up to the present.

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ELECTRON MICROSCOPIC OBSERVATIONS OF TOXOPLASMA 'NICOLLE ET MANCEAUX' GROWN IN TISSUE CULTURES (FIRST NOTE)

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(With Plate VII)

In the present paper we give the results of our study of Toxoplasma 'Nicolle et Manceaux' with the electron microscope. An attempt to study Toxoplasma with the electron microscope has been made by Bringmann & Holz (1953), who examined Toxoplasma gondii from the peritoneal fluid of mice.

From previous work (Guimaraes & Meyer, 1942; Meyer & de Oliveira, 1945) we know that Toxoplasma can be cultivated easily in tissue cultures of chick embryo cells. The parasites live and proliferate in the cell's cytoplasm where they multiply by binary division, filling the host cells until these are destroyed. Observations *in vivo* have shown that the parasites move towards the cells and penetrate them by their own activity. It has also been observed that outside the cells the parasites degenerate in a few hours if they do not find a new host cell.

MATERIAL AND TECHNIQUE

For the study in the electron microscope Toxoplasma was cultivated in subcutaneous and muscle tissue from 10 to 15-day-old chick embryos. When the tissue cultures showed many parasites they were transferred to coverslips which had been previously coated with Formvar 0·3%. The Maximow method was then used with double coverslips, the plasma was diluted 1:3, and care was taken to have the explant in contact with the Formvar-film. Some of the cultures were maintained in Roller-flasks. After 24–48 hr. of incubation the cultures were fixed in osmic acid vapours for 2 to 10 min. After the fixation they were thoroughly washed in distilled water. The plasma clot with the thick part of the culture was then removed and the Formvar-film with the remaining cells and parasites was transferred, using the technique of Porter, Claude & Fullam (1945), to the electron microscope grids. Here the preparations were shadowed with chromium and then observed in the 'Philips' electron microscope with 60 and 80 kV.

OBSERVATIONS

In the electron microscope one can see normal, i.e. unparasitized cells, cells with one or more parasites in the cytoplasm, and others which had just ruptured and freed the parasites. Groups of these can be found nearby, and free forms at a greater distance from the ruptured cell.

Many of the free parasites are too thick for a good penetration of the electron beam; this applies specially to the youngest forms, which have just left the host

cells (Pl. VII, fig. 1). They show the typical banana-form, and in the electron microscope it is seen that one end is larger than the other. Often a small transparent process is attached to the larger end which has no visible structure in the electron microscope. The whole body is covered by a very delicate, transparent mantle which shows no striations or myonemata. The parasite has neither flagella nor axonema-like filaments. However, in many of the extracellular forms thin membranes can be seen to appear in different parts of the body (Pl. VII, figs. 2, 3). They are apparently extensions of the thin mantle which covers the body. This mantle does not protect the parasites enough to maintain them in their original form. In the electron microscope most of them have an irregular surface and tend to become flat and large (Pl. VII, fig. 3). It is in these flattened forms that the central region, the region of the nucleus, sometimes becomes transparent. However, we are not sure whether these parasites are still in good condition, or whether they are already degenerating forms. We have therefore to postpone the interpretation of the structure in these regions until we can compare them with ultrathin sections of those parasites which are known to be in perfect condition but are too thick *in toto* for the penetration of the electron beam. Around the region of the nucleus we have never seen more than the dense and structureless masses which stain intensely with the osmic acid and which lead to a ring-like formation at one end of the parasite. Also, the intracellular forms which have been observed in these preparations are too dense for a good penetration of the electron beam (Pl. VII, fig. 6).

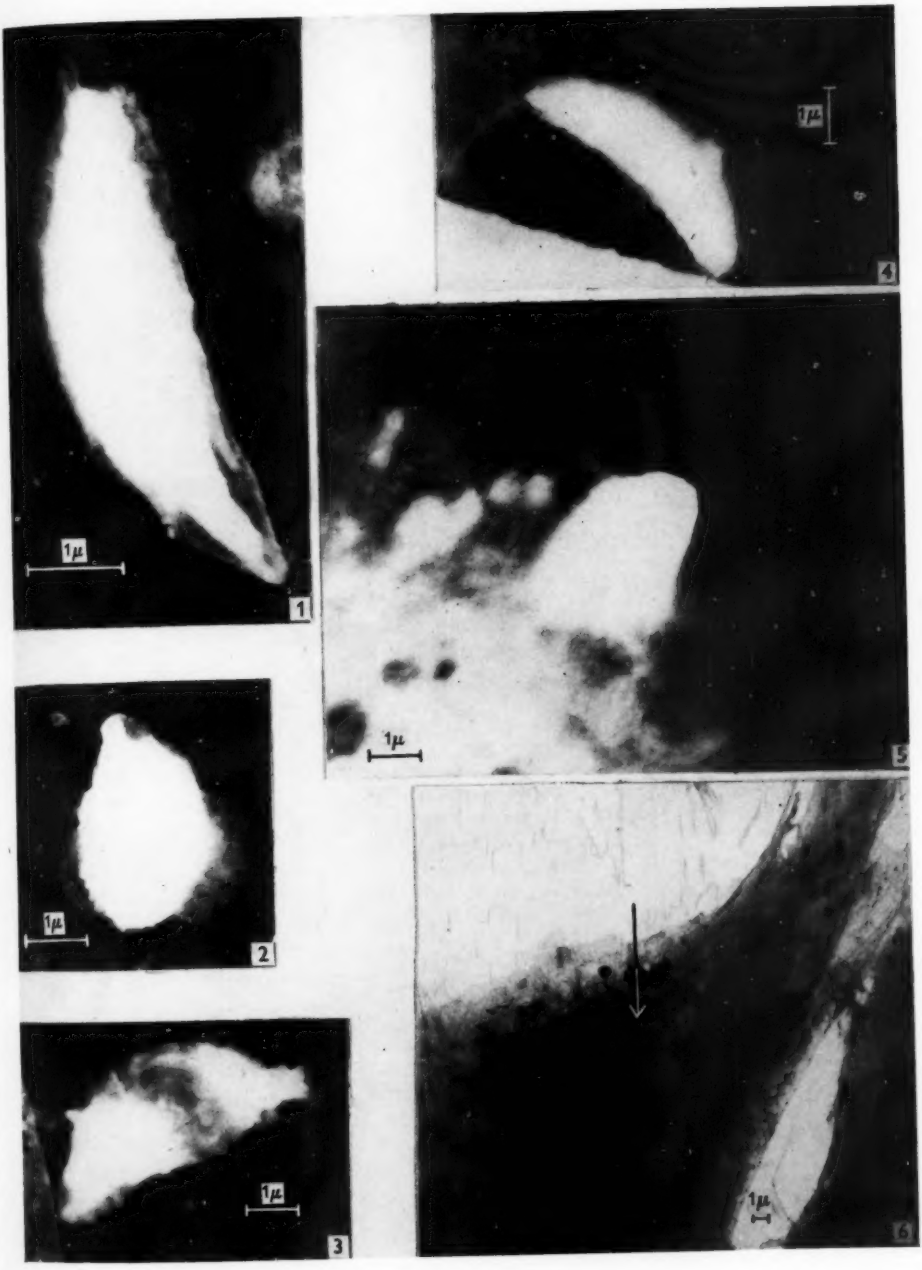
Pl. VII, figs. 4 and 5, give an idea of some aspects under which penetration of the cell may occur. From observations *in vivo* we know that the parasite forces itself through the cell wall, deforming its body during the act. The whole process is very rapid. Often we can see that a thin large membrane is extended, as if projecting itself for contact with the cell. Pl. VII, fig. 4 shows that small membrane-like processes make contact with the cell, while Pl. VII, fig. 5 shows one parasite half within and half still outside the cell. There seems to be no immediate reaction against the invader on the part of the cell, as far as it is possible to see this in the *in toto* preparations.

In a study of the parasitized cell we could see that the region of the cytoplasm around a parasite is lighter than in the rest of the cell. In the beginning of parasitism it is difficult to establish the limit between cytoplasm and parasite (Pl. VII, fig. 6). In more advanced stages, however, the cytoplasm is dissolved and a vacuole is formed around the parasites with a dark line of demarcation.

DISCUSSION

The observation of Toxoplasma in the electron microscope shows that the parasites are too thick for a good penetration of the electron beam. Those forms in which the central region has become transparent are possibly degenerating forms, the structure of which has to be considered with reserve and will be discussed later in comparison with ultrathin sections of the same material which are in preparation.

The extreme delicacy of the surface of the parasite may explain the fact that



(*Facing p.* 450)

Toxoplasma degenerates so rapidly outside the host cells and needs living cells for cultivation. The thin mantle which covers the body seems to give insufficient protection against external influences, and is not even strong enough to keep the shape of the parasite. It cannot be considered as a sheath but seems to be part of the cytoplasm itself, which may extend to form the membranes which we find in many of the free forms. Their role is probably for the locomotion of the parasite and its attachment to the tissue cells.

SUMMARY

Toxoplasma 'Nicolle et Manceaux' has been examined in the electron microscope in *in toto* preparations from tissue cultures.

The extracellular as well as the intracellular forms of the parasite are too thick for an adequate penetration of the electron beam, and the inner structure of Toxoplasma is not revealed in these preparations.

The whole parasite is covered by a very delicate, transparent mantle which may extend to form large membranes. They show no structure in the electron microscope. No sheath has been observed which protects the shape of the parasite. Most of the free forms have an irregular surface and tend to become flat and large.

Several pictures illustrate the way in which Toxoplasma penetrates the tissue cells. In the beginning of parasitism the limits between cytoplasm and parasite are difficult to recognize. The region of the cytoplasm around the parasite is gradually dissolved and a vacuole is formed.

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EXPLANATION OF PLATE VII

Fig. 1. Toxoplasma after having left the host cell. Body too thick for penetration of electron beam. A transparent mantle which shows no structure surrounds it. × 15,000.
Fig. 2. Free form of Toxoplasma with membranes at two sides. × 10,200.
Fig. 3. Toxoplasma with irregular surface, perhaps in beginning of degeneration. More transparent in central region than young forms. × 7500.
Fig. 4. Toxoplasma extending small processes towards cell. × 7500.
Fig. 5. Toxoplasma in act of penetration. No definite limits can be seen between cytoplasm of cell and parasite. × 7500.
Fig. 6. Toxoplasma in cell. No definite limits can be recognized between parasite and cytoplasm of cell. Area around parasite lighter than rest of cytoplasm. × 2100.

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STUDIES ON *ENTAMOEBA INVADENS* RODHAIN, 1934, *IN VITRO*, AND ITS RELATIONSHIP TO SOME OTHER SPECIES OF *ENTAMOEBA*

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(With 13 Figures in the Text)

CONTENTS

PAGE

I. Introduction 452
II. Factors affecting the growth and nutrition of *Entamoeba invadens in vitro* . 455
(1) Media and bacteria 455
(2) Starch 458
(3) Temperature 459
(4) The ingestion of particulate substances by the amoebae of *Entamoeba invadens* 460
(5) Discussion 461
III. The encystation of strains of *Entamoeba invadens in vitro* 463
(1) Experimental methods and results 463
(2) Discussion 467
IV. Factors affecting the survival of the amoebae and cysts of *Entamoeba invadens in vitro* 470
(1) The sensitivity of the amoebae to hydrochloric acid 470
(2) The survival of the amoebae at 8 and 37° C. 471
(3) The effect of oxygen on the amoebae 472
(4) The sensitivity of the cysts to hydrochloric acid and mercuric chloride 473
(5) The survival of the cysts at 8, 24 and 37° C. 474
(6) Discussion 474
V. The relationship of *Entamoeba invadens* to some other species of *Entamoeba* . 476
VI. Summary 479
References 479

I. INTRODUCTION

A disease of reptiles, comparable with amoebiasis in man and associated with a species of *Entamoeba* closely resembling *E. histolytica*, was first described by Ratcliffe & Geiman (1933, 1934), who hesitated to confer specific status on the reptilian *Entamoeba* until they had investigated its morphology and physiology more fully. Rodhain (1934) described an infection associated with an *Entamoeba* in reptiles dying in captivity, and to this organism he assigned the name *E. invadens*, considering that on both morphological and physiological grounds it could be distinguished from *E. histolytica*. Geiman & Ratcliffe (1936), after a detailed study of the organism which they isolated from infected reptiles, concluded that it was specifically distinct from *E. histolytica*, and consigned it to the species *E. invadens*, since it was probably identical with that described and named by Rodhain. Fantham & Porter (1950) considered that *E. invadens* was identical with *E. serpentis*, described from a reptile by Cunha & Fonseca (1917, 1918), and that

consequently the latter name had priority over *E. invadens*. Geiman & Ratcliffe (1936), however, believed that *E. serpentis* was probably synonymous with *E. testudinis*, but, as they point out, the organism described by Cunha & Fonseca will be difficult to identify, as these workers did not describe cysts, and the measurements of the amoebae were not given. Since the characters of *E. serpentis* are not completely known, there does not appear to be any justification for concluding that it is identical with *E. invadens*.

Infections with *E. invadens* have been described by several workers, and a list of the hosts which have been spontaneously or experimentally infected with this parasite is given by Hill & Neal (1954). Ratcliffe & Geiman (1938) were unable to infect specimens of *Anolis carolinensis*, *Aligator mississippiensis* or kittens with *E. invadens*, and Rodhain & van Hoof (1934, 1935) failed to produce infections in *Emys orbicularis* or in the frog, *Rana temporaria*. Geiman & Ratcliffe (1936) also attempted to produce amoebiasis in tadpoles by feeding them with cysts of *E. invadens*; a small proportion excysted, but apparently the amoebae were unable to survive in the small intestine. Intrarectal injections of cultures resulted in the establishment of amoebae in the lumen of the rectum, but as symptoms of disease were not observed, *E. invadens* appears to be non-pathogenic for the tadpole. From the available experimental evidence, susceptibility to infection by *E. invadens* appears to be limited to, but fairly widespread amongst, reptiles. Very little is known of the incidence of the parasite in nature.

Ratcliffe & Geiman (1938) have described in detail the pathological changes associated with experimental infections of *E. invadens* in *Natrix* spp., and they point out that in certain respects the symptoms associated with amoebiasis in reptiles are similar to those found in human amoebiasis. Compared with the lesions occurring in human amoebiasis, those in the reptilian disease are associated with relatively small numbers of amoebae. The chief difference noted is in the distribution of the lesions; lesions of the upper small intestine and stomach are peculiar to the disease in reptiles, in which, in addition, liver abscesses are more common than in human amoebiasis. Ratcliffe & Geiman suggest that these differences may be related to the feeding habits, and to certain anatomical and physiological differences between reptiles and man. In the majority of cases studied by Ratcliffe & Geiman, amoebiasis of reptiles ended in death, and it is not known if reptiles ever recover spontaneously from their infections, or if there is a condition in reptiles comparable to the 'carrier' state in man.

Morphologically, the amoebae and cysts of *E. invadens* are almost identical with those of *E. histolytica* (Geiman & Ratcliffe, 1936). Rodhain (1934) distinguished *E. invadens* from *E. histolytica* morphologically on the grounds of the extreme delicacy of the peripheral chromatin of the nucleus of *E. invadens*, and a difference in the appearance of the karyosome after fixation with Bouin's solution, but Geiman & Ratcliffe question the validity of these differences, which they themselves did not observe. These workers were able to find only minor morphological differences between the two organisms, namely a greater cytoplasmic density of the amoebae and a thicker cyst wall in *E. invadens* than in *E. histolytica*, and they considered that a more significant difference was to be found in the

nucleocytoplasmic ratios of the two organisms, 0·295 for *E. invadens* and 0·246 for *E. histolytica*. More recently, Baud & Borer (1952) have described certain differences in the ectoplasm of the two species, when examined for birefringence.

The resemblance between *E. invadens* and *E. histolytica* extends also to their life cycles; in all the details of encystation, excystation and metacystic development, *E. invadens* and *E. histolytica* are identical (Geiman & Ratcliffe, 1936), and a description of these processes in one species applies equally well to the other.

E. invadens has been grown successfully *in vitro* in media which support the growth of *E. histolytica* and many other species of *Entamoeba*, rice starch and bacteria being present in all the cultures. In contrast to *E. histolytica*, which multiplies only within the range 32–41° C. (Boeck & Drbohlav, 1925; Dobell & Laidlaw, 1926), *E. invadens* grows *in vitro* within the range 20–30° C. (Geiman & Ratcliffe, 1936). Both species complete all stages of their life cycles in culture, and their pathogenicity is not affected by growth *in vitro*, as cysts from cultures will infect and produce typical amoebiasis in the host.

As a result of the striking morphological resemblance between *E. invadens* and *E. histolytica*, and the marked similarity in the effects these parasites produce in their respective hosts, the question inevitably arises whether the two organisms are in reality biological races of the same species, rather than two separate species; there is certainly no justification for assigning them to different species on the grounds of morphology alone. Geiman & Ratcliffe (1936), in a discussion on the relation of *E. invadens* to *E. histolytica*, conclude that in view of the great physiological differences between their hosts, it is preferable to consider them as separate species than to regard *E. invadens* as a variety of *E. histolytica*.

A similar problem arose with regard to *E. histolytica* and *E. ranarum*, an intestinal parasite of the frog; of these two intestinal amoebae Dobell (1918) wrote 'there is no constant structural character which will permit of a distinction being drawn between these two species', but he concluded that they were separate species after he had failed to infect tadpoles with cysts of *E. histolytica*. *E. invadens* and *E. ranarum*, however, appear to be more closely related physiologically than *E. invadens* and *E. histolytica*, as both are found in cold-blooded vertebrates, but since an attempt by Geiman & Ratcliffe to infect tadpoles with *E. invadens* was unsuccessful, the present evidence, while not conclusive, suggests that *E. invadens* and *E. ranarum* may be separate species.

From the point of view of both morphology and pathogenicity, *E. invadens* and *E. histolytica* resemble each other more closely than do any other species of *Entamoeba*. Observations on the behaviour of *E. invadens in vitro* are few, and it was considered that a more detailed *in vitro* study of the organism might throw further light on its affinities with *E. histolytica*. The response of *E. invadens* to certain amoebicidal drugs *in vitro* has already been described (McConnachie, 1954), and the present paper describes the growth and encystation of certain culture strains of *E. invadens*, and the survival of amoebae and cysts under different conditions.

The following studies were made on strains of *E. invadens* in which the bacterial flora was known, namely strains A and C, growing with *Bacterium coli* (strain 15)

and an unidentified bacterium, organism *x*; strain D, growing with *Bact. coli* (strain FA′) and organism *x*; strain M, growing with *Bacillus megatherium* and organism *x*; and strain GV, growing with *Bact. coli* FA′ alone. These strains were derived from a strain of *E. invadens* originally isolated by Professor J. Rodhain. In a previous paper (McConnachie, 1954), the strain of *Bact. coli* in strain GV was designated A′, as it was then believed to be identical with the strain of *Bact. coli* isolated and named A′ by Dobell (1952). More recent examination of the organism in strains D and GV has shown that it is a mixture of both smooth and rough bacteria, and that whereas it produces acid from all the sugars fermented by *Bact. coli* A′, unlike A′ it does not produce gas; the strain of *Bact. coli* in strains D and GV has therefore been renamed FA′.

II. FACTORS AFFECTING THE GROWTH AND NUTRITION OF *ENTAMOEBA INVADENS IN VITRO*

(1) *Media and bacteria*

The strains of *E. invadens* grew readily, in the presence of starch (S), in several of the media which were first developed to support the growth of *E. histolytica* or other species of *Entamoeba*, and in all of which *E. histolytica* grows well; these media included the diphasic horse-serum–Ringer-eggwhite (HSre of Dobell & Laidlaw, 1926), whole egg–horse-serum–Ringer (Ehs of Dobell & Laidlaw), liquid horse-serum–Ringer (hs of Dobell & Laidlaw) and an infusion of egg-yolk in saline-phosphate (eysaph), prepared according to the method described by Balamuth & Sandza (1944). As in cultures of other species of *Entamoeba*, the amoebae were found only at the bottom of the culture tubes, where, presumably, the oxidation-reduction potential is more constantly maintained at a low level than in other regions of the tube.

In cultures containing organism *x*, *E. invadens* grew readily in hs + S, eysaph + S or HSre + S with *Bact. coli* (strains 15 or FA′) or *B. megatherium*; and in Ehs + S with *Bact. coli* (strains 15 or FA′). Growth also occurred in HSre + S, hs + S or eysaph + S, in the presence of *Bact. coli* FA′ alone.

Although *E. invadens* grew readily in the presence of organism *x* and either *Bact. coli* 15 or *B. megatherium*, its growth appeared to be less heavy in the presence of the latter species. The course of population growth in strains A and M was therefore studied in more detail, in order to determine the influence of the different bacteria on the growth of *E. invadens*.

Before the experiments were performed, the strains were maintained for several months in HSre, hs and eysaph, in order that they might become fully adapted to the media under investigation. In each experiment, two tubes, each containing 5 ml. of liquid media and approximately equal amounts of starch, were seeded with 5 drops of a 24 hr. broth culture of the relevant bacteria; 24 hr. later they were inoculated with approximately the same number of amoebae from a culture showing good growth. Each of the growth curves shown in Figs. 1–3 is based on the numbers of amoebae produced in two pairs of cultures.

The numbers of amoebae in the inocula were not determined; approximately

the same volume of culture medium containing amoebae was inoculated into each of the experimental tubes, and the comparative numbers of amoebae inoculated were calculated from estimations of the numbers of amoebae in the cultures from which the inocula were taken. The numbers of amoebae of strain A inoculated into hs and eysaph were several times greater than those of strain M inoculated into these media; cultures of the two strains in HSre medium, however, received approximately the same numbers of amoebae, and since in this medium the growth

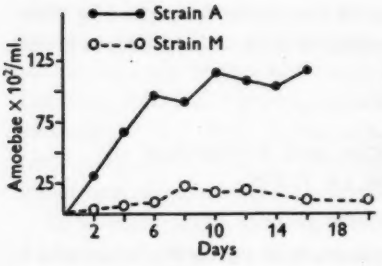

Fig. 1

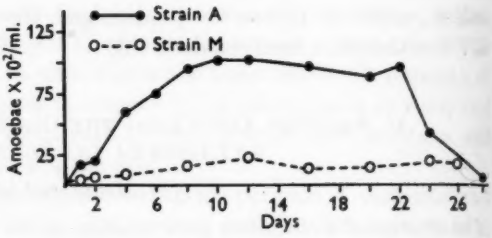

Fig. 2

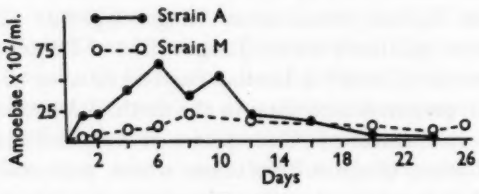

Fig. 3

Fig. 1. Growth curves of strains A and M in HSre + S. Uningested starch was still present in the cultures of strain M on the 20th day, but all the starch had been ingested in the cultures of strain A by approximately the 12th day.

Fig. 2. Growth curves of strains A and M in hs + S. All the starch had been ingested in the cultures of strain M by approximately the 20th day, and in those of strain A by approximately the 12th day.

Fig. 3. Growth curves of strains A and M in eysaph + S. A little uningested starch was present in the cultures of strain M on the 26th day, but all the starch had been ingested in the cultures of strain A by approximately the 12th day.

of the two strains was very different (Fig. 1), it was considered that in the other media also, differences in growth rate could be attributed to factors other than differences in the sizes of the inocula. Similarly, the growth of strain A in the different media was not related to the size of the inoculum; the numbers of amoebae inoculated into HSre medium were less than those inoculated into either hs or eysaph, but growth in HSre was consistently heavier than in the other media (Figs. 1–3).

The number of amoebae produced in an experimental culture was determined at intervals of 2–4 days by thoroughly mixing the culture and removing small amounts of the medium in order to count the amoebae in a haemocytometer; each

estimation, as recommended by Paulson (1932), was the mean value of six separate counts. Abnormal amoebae—i.e. granular and disintegrating amoebae—were not counted, but in old cultures it was sometimes difficult to assess by observation whether certain amoebae were still viable.

The general trends of growth, particularly in cultures of strain A, followed the classical pattern of bacterial growth (Figs. 1–3); the logarithmic phase of growth was succeeded, after 6–10 days, by phases of negative growth acceleration, maximum stationary growth and accelerated death. It was not possible to determine the numbers of amoebae in cultures growing in HSre after about 20 days, as the disintegration of the solid slopes in cultures of this age made it very difficult to count the amoebae; thus the growth curves in this medium do not show the phase of accelerated death. The growth of strain M was characterized by the absence of marked phases of logarithmic growth or accelerated death; the lag phase merged into a period in which growth attained its maximum level, and after which no marked decrease in the growth rate was observed during the period for which the cultures were examined.

The growth of strain M was essentially the same in each of the media studied, but was maintained at a slightly lower level in eysaph than in HSre or hs. The growth of strain A, however, was definitely influenced by the medium; in HSre, growth during the first 6 days was more rapid, and larger numbers of amoebae were produced than in hs, while in eysaph, multiplication was slower and fewer amoebae were produced. Table 1 shows the maximum yields of the two strains in each of the media.

Table 1. *The maximum yields of amoebae produced by strains A and M in different media*

Strain	Medium	Maximum yield of amoebae/ml.*
M	eysaph	1,950
M	hs	3,000
M	HSre	3,200
A	eysaph	5,550
A	hs	10,200
A	HSre	17,050

* Mean value of maximum yields of four cultures.

The growth of strain A was consistently heavier than that of strain M; since the growth of strain M varied little in the three media, the difference between cultures of the two strains was most marked in HSre and least in eysaph. It seemed possible that one of the factors responsible for the marked difference in the growth of the two strains might be a poorer degree of anaerobiosis in cultures of strain M; the growth of *B. megatherium* in the media studied was noticeably less than that of *Bact. coli* 15, and it was thought that the poorer bacterial growth in strain M cultures might fail to deoxygenize the medium completely. When, however, the growth of strain M in HSre medium was compared in 'aerobic' cultures (tubes with cotton-wool plugs) and 'anaerobic' cultures (tubes in which the oxygen was absorbed

with alkaline pyrogallol), no difference was detected in the multiplication rate or the numbers of amoebae produced under the different conditions.

It is not known to what extent the growth of *E. invadens* is influenced by organism *x*, but since this bacterium grew well in the cultures containing *B. megatherium*, the poor growth of *E. invadens* in the presence of *B. megatherium* and organism *x*, compared with its growth in cultures of *Bact. coli* 15 and organism *x* may be attributed to the effect of *B. megatherium*. It is probable that the growth of *E. invadens* is dependent on certain enzymes, nutrients or growth factors which are produced by *Bact. coli* 15, but only to a lesser degree or not at all, by *B. megatherium*, and a comparison of the metabolism of these bacteria might suggest certain of the growth requirements of *E. invadens*.

Cultures of strain M were presumably deficient in some substances necessary for optimum growth, and this may account for the different effects that the three media had on the growth of strains A and M. The more favourable flora of strain A allowed cultures of this strain to reach levels of growth which were then limited only by deficiencies in the medium; thus the rich HSre medium supported heavier growth than the less nutritious eysaph. The less favourable flora of strain M, on the other hand, was the primary factor limiting the growth of this strain, which was so inhibited by its flora that differences in the media had no detectable effect on its growth.

(2) *Starch*

The growth of *E. invadens* was markedly stimulated by the presence of starch in the medium; cultures of strain A have been maintained in HSre medium without starch for eleven subcultures, over a period of 11 weeks, but the growth in these cultures was very poor, and it is not known whether such starch-free cultures can be maintained indefinitely. In contrast, strains can be maintained indefinitely in HSre + S, in which abundant growth of the amoebae occurs.

It was found that in cultures of strain A in HSre the numbers of amoebae produced were related to the amount of starch in the cultures (Table 2 and Figs. 6–8); smaller yields were obtained from cultures in which all the starch had been ingested within a few days after inoculation than in those in which there was sufficient starch to last for several weeks.

Table 2. *The effect of the amount of starch on the numbers of amoebae produced in cultures of strain* A *in* HSre

Day by which all starch had been ingested	Maximum yield of amoebae/ml.	Day by which all starch had been ingested	Maximum yield of amoebae/ml.
4	1,150	8	7,100
6	1,550	10	12,750
6	1,700	12	17,600
8	4,600	16	30,400

Starch appears to have a stimulating effect on the growth of the amoebae in old cultures of strains A and M in HSre, hs or eysaph media. In cultures in which all the starch had been ingested, the numbers of amoebae continued to rise for a few

days during which the ingested starch was digested, but after that the numbers of amoebae decreased. When starch was added to cultures in which all the starch had been ingested and the numbers of amoebae were decreasing, an increased production of amoebae was observed, while in control cultures, without additional starch, the amoebae continued to decline in number. In cultures of strain A, this increase began immediately after the addition of starch, but in the strain M cultures, there was a lag of a few days before the number of amoebae began to increase (Figs. 4, 5).

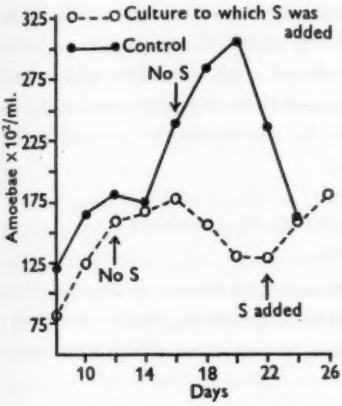

Fig. 4

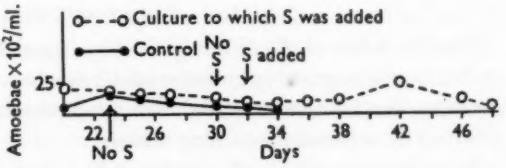

Fig. 5

Fig. 4. The effect of adding starch to a culture of strain A in HSre, in which all the starch had been ingested. S = starch. No S = day on which uningested starch could not be found.

Fig. 5. The effect of adding starch to a culture of strain M in eysaph, in which all the starch had been ingested. S = starch. No S = day on which uningested starch could not be found.

(3) *Temperature*

Cultures of *E. invadens* used throughout the present work were maintained at 24° C., but growth appeared to be equally heavy at 28° C. or 30° C.; strains could also be maintained, apparently indefinitely, at room temperature (16–20° C.), but growth was less heavy at these lower temperatures than at 24° C. When cultures were placed at 37° C., it was observed that although the amoebae survived for several days, there was no evidence that they multiplied, and subinoculations into new medium at 37° C. were invariably negative.

An attempt was made to 'train' the amoebae of *E. invadens* to grow at temperatures higher than those at which growth normally occurred. A substrain of strain C was accordingly maintained in hs + S at gradually increasing temperatures This substrain was first maintained for 3 subinoculations at 28° C., followed by twelve subcultures at 30° C., over a total period of 4 months, during which no appreciable decrease in the intensity or rate of growth was observed. It was then maintained at still higher temperatures for a further period of 10 months. At 33° C., subinoculations were made at intervals of 4–6 days, and growth was almost as heavy as at 30° C., but when, after twelve subcultures at 33° C., the strain was

maintained at 34–35° C., very poor cultures were produced; although only a few amoebae were found in these cultures, they were normal in appearance, being motile and containing starch. The strain was maintained at temperatures varying between 33·5 and 35° C., by subinoculation at intervals of approximately 5 days, for a total of forty-eight subcultures; in the later cultures, many abnormal amoebae were found, and the strain eventually died out and could not be recovered even when inoculated into medium maintained at 24° C. During the 8 months in which the strain was kept at 33·5–35° C., it was maintained only with considerable difficulty, and cysts were never found. When subinoculations were made from cultures growing at these temperatures into medium at 37° C., such cultures did not survive longer than did similar cultures inoculated with amoebae which had been maintained at 24° C. Thus the amoebae retained their original sensitivity to temperature, even after they had been maintained for a considerable period at the maximum temperature at which they would grow.

(4) *The ingestion of particulate substances by the amoebae of* Entamoeba invadens

The amoebae of *E. invadens* readily ingest bacteria and starch grains *in vitro*, and in actively growing cultures each amoeba contains numerous grains of starch; glycogen can be demonstrated, using iodine solution, in amoebae which have been growing in cultures containing starch.

In cultures in which all the starch had been ingested, it was noticed that when the amoebae had digested their starch, they readily ingested cysts; in cultures growing in HSre and eysaph, for instance, many amoebae were observed containing at least one cyst, and some had as many as six to seven ingested cysts. The ready ingestion of cysts by the amoebae of *E. invadens* in cultures depleted of starch, was also observed by Geiman & Ratcliffe (1936), who, however, did not observe the ingestion of more than three cysts by one amoeba. Rodhain & van Hoof (1936) reported that the ingestion of cysts by the amoebae of this species was rare.

Conflicting reports have been made concerning the ability of the amoebae of *E. invadens* to ingest erythrocytes. Thus Geiman & Ratcliffe (1936) were unable to identify host erythrocytes within amoebae infecting reptiles, or to demonstrate the ingestion of human or horse erythrocytes, but they give no details of the conditions in the cultures to which the red cells were added. Rodhain & van Hoof (1936) demonstrated that in cultures containing little or no starch, a certain number of amoebae ingested human red cells or the nuclei of reptile erythrocytes. The effect of adding different kinds of erythrocytes to cultures of *E. invadens* was therefore investigated.

Defibrinated chick blood was added to a culture in which all the starch had been ingested; no red cells had been ingested when the culture was examined 2½ hr. after the addition of the blood, but many erythrocytes were attached to the posterior ends of the amoebae. Twenty-four hours later, the erythrocytes had shrunk to about half their original size, and one or two had been ingested by a few amoebae. Similar observations were made when whole frog blood was added to a

culture; no normal erythrocytes were ingested, but after 24 hr. a few shrunken red cells had been ingested by a small proportion of the amoebae. When whole rat blood was added to cultures in which all the starch had been ingested, a few amoebae were found with ingested erythrocytes 1 hr. after the addition of the blood; some amoebae contained many red cells, while others had only one. The majority of amoebae, however, did not ingest any erythrocytes, and 24 hr. later, when there were still many normal erythrocytes in the medium, none was found in the amoebae. In a second experiment, approximately half the amoebae in the culture had ingested rat erythrocytes 2 hr. after their addition to the culture; some amoebae contained only one, but the majority had two or three, and a few amoebae were observed with seven to ten erythrocytes. Eighteen hours later, there were still many amoebae containing two to four erythrocytes.

(5) *Discussion*

The course of population growth of *E. invadens* in cultures which showed optimal growth (i.e. strain A) appears to be essentially the same as that in cultures of *E. histolytica* which have been investigated by methods similar to those used in the present study. Thus Balamuth & Howard (1946) and Balamuth & Wieboldt (1951) showed that the growth curves of *E. histolytica*, growing with mixed or monobacterial flora, followed the classical bacterial pattern, and that, in general, higher yields were produced in HSre than in eysaph media. Balamuth & Wieboldt compared the growth of strains of *E. histolytica* growing with tri- and monobacterial flora in eysaph + S; growth was consistently heavier in strains accompanied by three species of bacteria, but strains of *Bacterium* (= *Escherichia*) *coli* (*E. coli communior* or *E. coli anaerogenes*) were more favourable for growth than any other single species tested. Similarly, optimal growth of *Entamoeba invadens* was obtained in the presence of a strain of *Bact. coli*. The difference in the growth of *E. histolytica* in HSre and eysaph media in the presence of a mixed bacterial flora (Balamuth & Howard, 1946) was not so marked as that found for *E. invadens* in these two media, in the presence of *Bact. coli* 15 and organism *x*. Balamuth & Wieboldt also found that the oxidation-reduction potentials produced in the different cultures were related to the growth-promoting properties of the different bacteria; bacteria which produced the highest degree of anaerobiosis supported the heaviest growth of amoebae. Although *B. megatherium* probably produced a lesser degree of anaerobiosis than did *Bact. coli* 15, the poor growth of *E. invadens* in the presence of the former species appeared to be correlated with some factor other than the degree of anaerobiosis produced. As pointed out by Dobell (1952), however, the growth-promoting properties of different bacteria are dependent on different factors.

The outstanding difference between the growth of *E. invadens* and *E. histolytica*, which is demonstrated by a comparison of their growth curves, is the rate at which they grow. Cultures of *E. histolytica* attain their maximum growth at approximately 2–3 days after inoculation, and by the 5th day or earlier, have reached the phase of accelerated death. Maximal growth in cultures of *E. invadens* was not reached until the 6th–10th day, and although in eysaph medium the number of

amoebae began to decline by approximately the 11th day, in HSre or hs media it was maintained for much longer at a stationary level. It may be presumed that the more rapid growth of *E. histolytica* results in a more rapid utilization of the nutrients in the medium, and this may partly explain why cultures of this species die several weeks before those of *E. invadens*; in addition, the more rapid growth of the bacteria in cultures of *E. histolytica* would produce an unfavourable environment more rapidly than in cultures of *E. invadens*.

The difference in the growth rates of the two species may be correlated with their different optimum temperatures for growth. *E. invadens* grows at temperatures between approximately 16 and 35° C., and *E. histolytica* between 32 and 41° C.; this appears to be a fundamental difference between the two organisms. The metabolism of *E. invadens* is presumably dependent on enzymes which can function between the range 16–35° C., with an optimum temperature for enzymic activity at approximately 24° C.; these enzymes must be physiologically distinct from those of *E. histolytica*, as an attempt to improve the growth of *E. invadens* above 35° C. was not successful, which suggests that the enzymes of this organism cannot adapt to function at temperatures at which those of *E. histolytica* are most active.

The effect of starch on the growth of *E. invadens* and *E. histolytica* appears to be identical; not only is the growth of both species enhanced by the presence of starch, but, as was shown for *E. histolytica* (Spingarn & Edelman, 1950), the yield of amoebae is related to the amount of starch in the culture. In addition, starch has a stimulating effect on the growth of the amoebae of both species in old cultures which have become depleted of their starch; Balamuth & Howard (1946) showed that this stimulating effect of starch on the amoebae of *E. histolytica* was due to a specific effect on the amoebae, as the numbers of bacteria continued to decline, even after the addition of starch.

The amoebae of *E. invadens* and *E. histolytica* readily ingest starch and bacteria *in vitro*, and, as described for *E. histolytica* by Cleveland & Sanders (1930), cysts may also be ingested. The affinity for host erythrocytes appears to be an outstanding difference between the two species. It has long been known that in cases of amoebic dysentery, the amoebae of *E. histolytica* ingest human erythrocytes, and Boeck & Drbohlav (1925) were the first workers to show that erythrocytes were also ingested *in vitro*, some amoebae being observed with as many as fifteen red cells; in contrast, *E. invadens* apparently does not ingest the erythrocytes of infected reptiles, and the ingestion of whole reptile erythrocytes *in vitro* has not been demonstrated convincingly. In the present experiments, normal chick or frog erythrocytes were not ingested *in vitro*, but those of rat were, and although *E. invadens* does not appear to ingest erythrocytes as readily, or in such large numbers as does *E. histolytica*, the ability to ingest them is found in both species. It is possible that size may be a factor determining the ingestion of erythrocytes by *E. invadens*; thus the small rat corpuscles were ingested more readily than those of chick or frog, which were ingested only after they had decreased in size. Chick and frog erythrocytes are of approximately the same size as those of reptiles, and the large size of the latter may explain why they are not readily ingested by *E. invadens* in the host.

III. THE ENCYSTATION OF STRAINS OF *ENTAMOEBA INVADENS IN VITRO*

(1) *Experimental methods and results*

It had been observed that in the original strain of *E. invadens* maintained at the Molteno Institute, cysts were produced when the strain was transferred to starch-free medium, after it had been grown in the presence of starch; this is in contrast to the behaviour of the strain of *E. histolytica* studied by Dobell (1928), which produced large numbers of cysts when transferred to HSre + S or Ehs + S, after being maintained in starch-free medium. Hitherto, the factors affecting the encystation of *E. invadens* have not been investigated, although Ratcliffe (1934) found that a gastric mucin medium favoured the production of cysts. A fuller investigation of the factors affecting the encystation of *E. invadens* seemed of interest.

The encystation of strains A and M was studied in HSre, hs and eysaph media, by determining the numbers of amoebae and cysts produced in cultures of the two strains in the different media. Before making observations on encystation, the strains were maintained for several months in each of the media under investigation. For each test, approximately the same numbers of amoebae were inoculated into two tubes containing 5 ml. of medium and seeded 24 hr. previously with five drops of a 24 hr. broth culture of the relevant bacteria. The numbers of amoebae and cysts were determined, as in the population studies, by counting in a haemocytometer. In Figs. 6–12 the number of amoebae and the percentage of cysts in the population are plotted on the same graph, against the age of the culture, and each graph is based on the determinations obtained from two cultures inoculated at the same time with approximately the same number of amoebae. Approximately the same amount of starch was added to each culture in any one experiment, and the time noted when uningested starch grains could not be found in the cultures; in general, the starch disappeared on the same day, or within 2 days of each other, from pairs of cultures which received similar inocula. No attempt was made to add identical amounts of starch in separate experiments.

The production of cysts was found to differ not only in the different media, but also in the two strains; the results obtained are considered below, for each strain, in each medium.

Strain A in HSre + S (Figs. 6–8)

An increase in the production of cysts was always correlated with the depletion of starch in the cultures; the percentage of cysts began to increase a few days before all the starch grains had been ingested, and continued to rise after the starch had disappeared from the medium, and while the numbers of amoebae containing starch rapidly decreased. This increase in the percentage of cysts was due to a real increase in the proportion of cysts produced; in the cultures illustrated in Figs. 6 and 7 the increase in the percentage of cysts after the disappearance of starch could be explained, in part, by the simultaneous fall in the numbers of amoebae, but in these cultures, and in those illustrated in Fig. 8, the initial rise in cyst production

began while the numbers of amoebae were still increasing. In addition, it can be seen that in some cultures, the numbers of amoebae as well as the percentage of cysts, continued to rise after all the starch had been ingested (Fig. 8). The amoebae appeared to form large numbers of cysts when the starch in the cultures became scarce, and continued to encyst after their ingested starch had been digested.

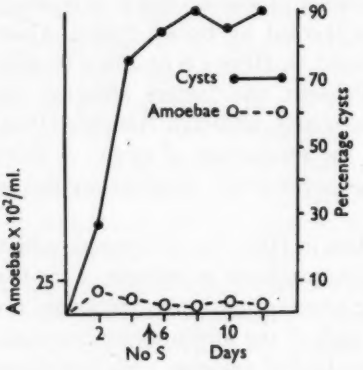

Fig. 6

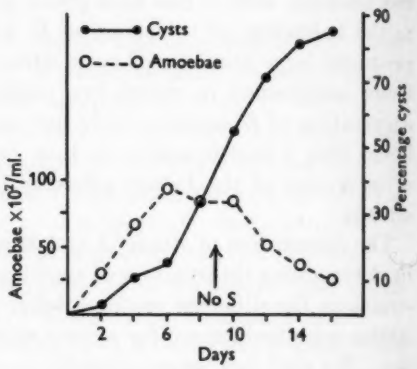

Fig. 7

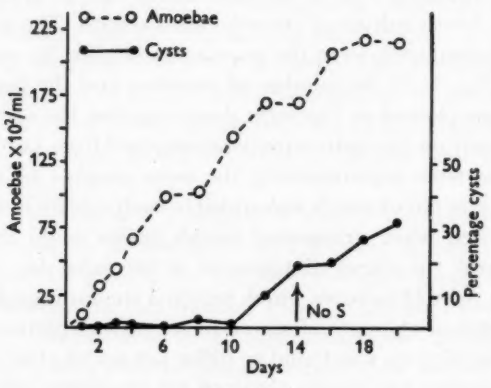

Fig. 8

Figs. 6–8. The production of cysts in cultures of strain A in HSre + S. No S = day on which uningested starch could not be found.

Strain A was maintained in starch-free HSre for seven subcultures, made at intervals of 7 days, and then inoculated into HSre + S. Mass encystation occurred in this culture only when all the starch had been ingested, just as it did in cultures inoculated with amoebae which had been growing in the presence of starch.

The proportion of cysts formed was also correlated with the age of the culture at the time when all the starch had been consumed by the amoebae (Table 3); maximum cyst formation occurred in those cultures in which the starch had disappeared by the 6th–8th day. When strain A was inoculated from HSre + S into starch-free medium, the starch present in the inoculum was rapidly consumed by

the multiplying amoebae; large numbers of cysts were produced, and only a few amoebae remained unencysted. During the first days after inoculation, the amoebae multiplied more rapidly than at other phases of their growth, and if the starch available to the amoebae became scarce during this period of rapid multiplication, then conditions most favourable for encystation were produced.

Table 3. *The effect of the age of the culture, at the time of the disappearance of starch, on the encystation of strain A in HSre+S*

Age in days, when starch disappeared	Percentage cysts on day when starch disappeared	Age in days, when starch disappeared	Percentage cysts on day when starch disappeared
4	70	8	84
6	80	10	46
6	82	12	19
8	56	16	3

In practice, it was found that the largest numbers of cysts were obtained by inoculating amoebae into HSre medium containing sufficient starch to last for about 8 days; the percentage of cysts in the population was rather less than in cultures in which the starch disappeared earlier, but the greater numbers of amoebae produced in the 8-day period of multiplication resulted in a greater yield of cysts.

No detailed observations were made of the pH in the cultures of strain A in HSre medium, but the range was from approximately 6·0 at the time of inoculation of the amoebae, to slightly over 7·0 at the end of the tests.

Strain A in Ehs+S

Although the encystation of strain A in Ehs+S was not studied in detail, it was observed that cysts were formed under the same conditions as in HSre+S; large numbers of cysts were produced as the starch became depleted. In addition, the onset of encystation was not influenced by previous maintenance of the strain in starch-free Ehs; after cultures had been maintained in starch-free Ehs for eight subcultures, large numbers of cysts were produced in Ehs+S only as the starch became depleted.

Strain A in hs+S (Fig. 9)

In this medium, a small proportion of cysts was formed during the early growth of the cultures, and thereafter there was no increase in the proportion of cysts produced. The disappearance of starch from the cultures had no influence on encystation, although the numbers of amoebae were maintained at a constant level for some time after all the starch had been ingested. The rapid rise in the percentage of cysts by the 24th–28th days was not due to an increased production of cysts, but to the fall in numbers of amoebae over this time.

Observations on the production of cysts in hs medium buffered with phosphate to give media of different pH, showed that within the range studied (6·0–7·6), at which good growth of amoebae occurred, pH had no influence on encystation.

Strain A in eysaph + S

The encystation of strain A in eysaph + S was variable, but was never correlated with the depletion of starch in the medium. In some cultures, in which the growth of amoebae was only slightly less than in cultures of strain A in hs + S, the cysts represented only a small proportion (less than 5 %) of the population, whereas in others, in which comparatively slight growth of amoebae occurred, as many as 60 % cysts were found. The pH range in all these cultures was 7·0–7·5.

When a strain which had been maintained in dilute eysaph + S (one-quarter the normal strength), in which growth was poor, was inoculated into full-strength eysaph + S, which supported a heavier growth of amoebae, the production of cysts was no greater than in strains which had been maintained in the full-strength medium. A transfer from a less to a more nutritious medium, therefore, had no effect on encystation.

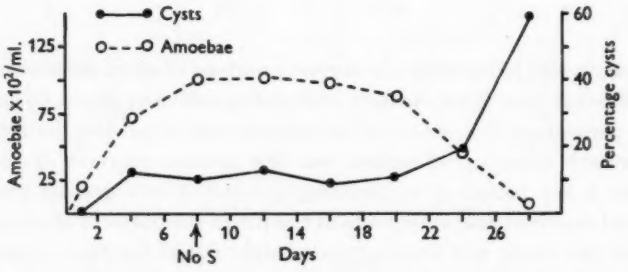

Fig. 9. The production of cysts in cultures of strain A in hs + S. No S = day on which uningested starch could not be found.

Strain M in HSre + S (Figs. 10, 11)

Unlike strain A grown in HSre + S, there was no marked correlation between the depletion of starch and the formation of cysts when strain M was grown in HSre + S. Comparatively large numbers of cysts were formed 6–8 days after the inoculation of the cultures, while there was still plenty of uningested starch present.

Strain M in hs + S (Fig. 12)

Few cysts were formed while starch was present in the medium, and the marked increase in the percentage of cysts produced was correlated with the disappearance of the starch. The increase in the percentage of cysts was due to an actual increase in the numbers of cysts formed, as the numbers of amoebae were maintained at approximately the same level, even after all the starch had been ingested.

Strain M in eysaph + S

The numbers of amoebae and cysts produced by strain M in eysaph + S were too small for graphical illustration. A small number of cysts was regularly produced, however, rising to approximately 7·5 % of the total population, but encystation was not influenced by the disappearance of starch from the medium.

To summarize the results, in strain A grown in HSre + S or Ehs + S, and to a lesser degree, in strain M grown in hs + S, an increase in the formation of cysts was correlated with the depletion of starch in the medium; but in strain A grown in hs + S or eysaph + S, and in strain M in HSre + S or eysaph + S, no such correlation was observed.

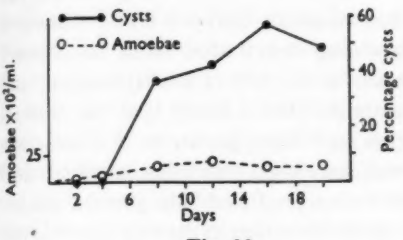

Fig. 10

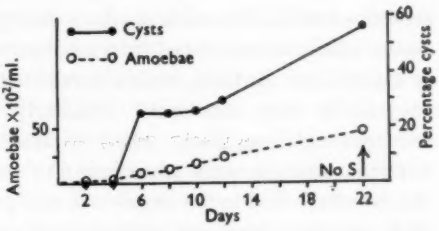

Fig. 11

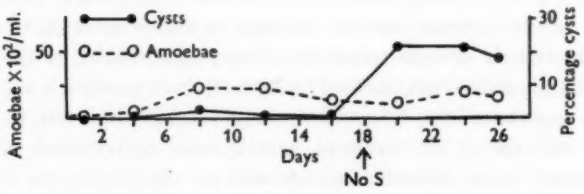

Fig. 12

Figs. 10, 11. The production of cysts in cultures of strain M in HSre + S. A small amount of uningested starch was still present on the 20th day, in the cultures illustrated in Fig. 10. No S = day on which uningested starch could not be found.

Fig. 12. The production of cysts in cultures of strain M in hs + S. No S = day on which uningested starch could not be found.

(2) *Discussion*

The strains of *E. invadens* studied produced cysts in HSre, Ehs, hs and eysaph media, but mass encystation occurred only in HSre or Ehs media; in the other media, the number of cysts produced was maintained at a relatively constant level, and in general represented a comparatively small proportion of the whole population.

Both strains A and M formed large numbers of cysts when grown in HSre medium. It has already been shown that, of all the media investigated, HSre supported the most prolific growth of *E. invadens*; although strain A grew almost as well in hs as in HSre, cultures in HSre consistently produced larger numbers of amoebae than those in the wholly liquid medium, while the growth of strain M in the two media was approximately the same. In contrast, when strains of *E. invadens* were grown under conditions permitting of only poor growth, cysts were not formed (see p. 460). Maintenance in a medium favourable to the growth of amoebae is, therefore, of importance in the production of large numbers of cysts of *E. invadens*.

A relationship between growth and encystation has been demonstrated many times in strains of *E. histolytica*. Cleveland & Sanders (1930) were the first to show

that mass encystation occurred only after a period of heavy growth and, since then, more detailed studies have stressed the importance of this factor. It now appears that in strains of *E. histolytica* an acceleration in growth rate, rather than the maintenance of prolific growth, is required for mass encystation, and Chang (1942) has suggested that this may account for the gradual loss of ability to encyst observed in strains of *E. histolytica* maintained in media containing starch; the growth of strains in such media is heavy, but attains a uniform level, whereas a strain which is inoculated into medium containing starch after being maintained in starch-free medium, shows a rapid increase in its rate of multiplication, and encystation may then occur. Similarly, Balamuth (1951) found that encystation occurred most regularly when strains which had been grown in a dilute, less nutrient medium were transferred to an enriched, highly nutrient medium, and this he attributed to the acceleration in growth thus produced. The present studies on *E. invadens*, however, indicate that such an acceleration in growth rate was not necessary for the encystation of the strains investigated. Thus when strains which had been grown in a dilute egg-yolk medium were transferred to a similar but more concentrated medium, there was no increase in the proportion of cysts formed, compared with that in strains which had been maintained in the concentrated medium; similarly, strains maintained in HSre did not produce a higher proportion of cysts when inoculated into HSre + S than did strains which had been maintained in HSre + S. Strains of *E. invadens*, which were maintained in HSre + S for 8 months (twenty-three subcultures), showed no diminution in their ability to produce cysts, and it appears that this organism is able to encyst in large numbers if it is growing under conditions which are favourable for its growth. Everritt (1950), in studies on the relationship between growth and encystation in strains of *E. histolytica*, has produced data which indicate that in one strain the percentage of cysts formed was directly proportional to the numbers of amoebae produced; no such relationship was found in the strains of *E. invadens*.

The results obtained clearly show that in cultures of strain A in HSre + S, an increased production of cysts was related to the depletion of starch in the medium, and a high proportion of cysts was formed even in cultures in which all the starch had been ingested before large numbers of amoebae were produced; depletion of starch was also correlated with an increased production of cysts, but to a lesser degree, in cultures of strain M growing in hs. No reference has been found in the literature to a correlation between encystation and depletion of starch in strains of *E. histolytica*; such a correlation, if it exists, might not be as marked as in strains of *E. invadens*, owing to the dependence of encystation in *E. histolytica* on an acceleration in growth rate.

It is perhaps of interest to note that the two organisms are exposed to very different conditions in their respective hosts; *E. histolytica* lives in the gut of mammals, which feed regularly, and *E. invadens* in the gut of reptiles, which feed sporadically and which, during hibernation, can live for long periods without food. The cysts of *E. histolytica* are normally formed in the lumen of the gut, and are regularly produced by infected persons showing no symptoms of the disease. Observations on the formation of cysts of *E. invadens* have been made only on

reptiles which died of their infections, and in which cysts were found in faecal material and in intestinal and liver lesions (Ratcliffe & Geiman, 1938; Geiman & Ratcliffe, 1936; Hill & Neal, 1954); these infected reptiles took no food after being inoculated with cysts. The formation of the cysts of *E. histolytica* in the host does not appear to be a response to adverse conditions, as was postulated in the past by some workers; it is possible, however, that the onset of adverse conditions, namely a decline in the food supply of the host, which may affect the bacterial flora on which the amoebae in the lumen of the gut feed, may induce cyst formation in *E. invadens*, the normal environment of which is more variable than that of *E. histolytica*. It is of interest in this connexion that Dobell (1918) found cysts of *E. ranarum* in the faeces of infected frogs only in the breeding season, and he suggested that the amoebae of this species encysted only in the winter and early spring, when the host ceased to feed. The factors affecting the encystation of *E. ranarum in vitro* have not been investigated in detail, but Sanders (1931) reported that this species encysted in several kinds of diphasic media containing starch, the proportion of cysts increasing with the age of the cultures.

The concomitant bacterial flora plays an important part in the formation of cysts of *E. histolytica*. Even when little was understood of the factors controlling encystation, Dobell (1928) and Cleveland & Sanders (1930) stressed the importance of maintaining a constant flora, if cysts were to be produced regularly. Several workers (Chinn, Jacobs, Reardon & Rees, 1942; Dobell, 1952; Neal, 1952) have isolated anaerobic spore-bearing bacteria which specifically induced encystation in certain strains of *E. histolytica*, but as Balamuth (1951) reports that encystation occurred in a strain of *E. histolytica* which was growing in the presence of three species of bacteria, none of which was an anaerobic spore-bearer, the presence of such a bacterium is not always essential for encystation in *E. histolytica*. Similarly, the presence of anaerobic spore-bearers was not necessary for the encystation of the present strains of *E. invadens*; cysts were produced in the presence of *Bact. coli* FA′ alone (strain GV), and in the presence of organism *x* (which does not appear to form spores) and either *Bact. coli* FA′ (strain D), *Bact. coli* 15 (strain A), *B. megatherium* (strain M) or *Achromobacter* sp.

Chang (1942, 1946) has correlated encystation in *E. histolytica* with certain conditions of pH and oxidation-reduction potential, but, as he suggested, it is possible that the change in oxidation-reduction potential which accompanied encystation was a reflexion of other conditions associated with the formation of cysts, rather than the actual factor inducing encystation. No evidence was obtained from the present studies that pH was a factor of importance in inducing encystation in *E. invadens*; it is possible that any influence that pH may have on the formation of the cysts of *E. histolytica* is indirect, and due primarily to the effect of changes in pH on the growth of the amoebae.

It has already been pointed out that the proportion of cysts produced in strain A of *E. invadens* in HSre medium was related to the age of the cultures at the time when the starch disappeared, and it may be concluded that mass encystation in *E. invadens* is favoured by the depletion of starch in comparatively young cultures growing under conditions which are potentially capable of supporting prolific

growth of amoebae, that is, a favourable medium and bacterial flora. Mass encystation in *E. histolytica* is dependent on an acceleration in amoebic growth rate, and, in certain strains, on the presence of anaerobic spore-bearing bacteria. It is possible that a certain condition of the nutrition of the amoebae is an essential prerequisite for the encystation of intestinal amoebae; this condition may be most readily attained in *E. histolytica* by rapid multiplication in the presence of certain bacteria, and in *E. invadens*, by a decline in the food available to actively multiplying amoebae.

IV. FACTORS AFFECTING THE SURVIVAL OF THE AMOEBAE AND CYSTS OF *ENTAMOEBA INVADENS IN VITRO*

(1) *The sensitivity of the amoebae to hydrochloric acid*

Whereas certain strains of *E. histolytica*, for instance the monobacterial strain obtained by Dobell (1947, 1952), never form cysts, non-encysting strains of *E. invadens* are not known. Thus, in order to test the sensitivity of the amoebae of this species, a method was required by which amoebae could be obtained free of cysts. Dobell (1927) produced cyst-free cultures of *E. histolytica* by initiating cultures from cysts; after excysting, the amoebae multiplied rapidly for a short time without encysting, and as the cysts of this species have a relatively short life at 37° C., there was a short period after all the viable cysts had hatched, during which new cysts were not formed. This method was not found suitable for obtaining cyst-free cultures of *E. invadens* because some of the amoebae arising in cultures initiated from cysts encysted before all the original cysts had hatched, and at no time were such cultures found to contain only amoebae.

A medium was therefore sought which supported a regular but slight growth of *E. invadens*, in the hope that cysts would not be formed when the growth of the amoebae was depressed. Balamuth (1951) reported that the growth of a strain of *E. histolytica* maintained in eysaph of half the normal strength was markedly less than that of strains growing in the full-strength medium. Cultures of *E. invadens* maintained in half-strength eysaph appeared to grow almost as well as in the full-strength medium, but growth was considerably reduced in medium of one-quarter the normal strength (1/4 eysaph). After strain M had been maintained in 1/4 eysaph + S for eight subcultures, made at intervals of approximately 3 weeks, cultures were carefully examined, and cysts were never found. When amoebae from these cultures were treated with N/20-HCl for 1 hr., and then inoculated into culture medium, cultures of the organism were never obtained, whereas similar treatment of samples known to contain cysts invariably produced cultures of amoebae; thus strains maintained in the dilute medium did not appear to form cysts. Cultures of strains M and D which had been maintained for several months in 1/4 eysaph + S were therefore used for the studies on the survival of amoebae; no difference was observed in the response to acid of amoebae from the two strains.

Amoebae from four to six cultures of strains M or D in 1/4 eysaph + S were pooled in Ringer's solution and concentrated by centrifugation. The deposit of amoebae and starch was then suspended at room temperature in N/20-HCl, which,

after varying intervals, was neutralized with N/5-$NaHCO_3$, neutral red being used as an indicator of pH. As a control, amoebae were suspended in Ringer's solution containing neutral red. At the end of the test, the amoebae in each tube were centrifuged down and inoculated into medium containing starch and previously seeded with the relevant bacteria. These cultures were examined at weekly intervals for periods up to 3 weeks, in order to determine whether any viable amoebae were present in the inocula. It was found that the amoebae were generally killed by exposure to N/20-HCl for 10 min., although in one experiment they survived treatment for 30 min. but not for 1 hr. (Table 4).

Table 4. *The sensitivity of the amoebae of* Entamoeba invadens *to* N/20-HCl, *at room temperature* (*approximately* 16–20° C.)

	Suspending fluid	Time	Survival*
Expt. 1	Ringer	1 hr.	+
	N/20-HCl	10 min.	0
Expt. 2	Ringer	40 min.	+
	N/20-HCl	10 min.	0
Expt. 3	Ringer	40 min.	+
	N/20-HCl	10 min.	0
Expt. 4	Ringer	1 hr.	+
	N/20-HCl	10 min.	+
	N/20-HCl	30 min.	+
	N/20-HCl	1 hr.	0

* Determined by subculture.

(2) *The survival of the amoebae at* 8 *and* 37° C.

Few observations have been made on the effect of temperature on the survival of *E. invadens*. Brumpt (1939) considered that the amoebae of this species were killed by low temperatures, at which the cysts survived; he based this conclusion on the observation that amoebae disappeared from the cloacae of infected reptiles which were maintained for 7 days at 6° C., but reappeared when the reptiles were replaced at 25° C. Since Brumpt's assumption that these amoebae were derived from cysts is hardly justified, a more detailed investigation was made on the survival of amoebae *in vitro* at 8 and 37° C.

The survival of amoebae at different temperatures was determined by inoculating the pooled amoebae obtained from four to six cultures of strains M or D, growing in 1/4 eysaph + S, into tubes of medium (hs + S or eysaph + S) which were incubated at 8, 24 or 37° C.; these cultures were examined at regular intervals, and when living amoebae could no longer be found, subinoculations were made into fresh medium maintained at 24° C., in order to determine if there were any viable amoebae in the original cultures.

The amoebae used in these experiments were obtained from cultures in which, it was believed, cysts were never formed, but in each test the absence of cysts from the inocula was verified by determining the resistance to HCl of the amoebae obtained from the pooled cultures. At the time when the amoebae were inoculated

into the media maintained at the different temperatures, a portion of the inoculum was treated with N/20-HCl for 1 hr., as previously described; in no experiment were cultures of amoebae obtained from such acid-treated amoebae, and since it was known that cysts would withstand such treatment with acid (see above), it was unlikely that cysts were introduced into the cultures of amoebae which were incubated at 8 and 37° C.

It was found that the amoebae could survive for 7 days at 37° C., and for at least 7 days at 8° C., though in two out of three experiments they survived for considerably longer periods at this temperature (Table 5).

Table 5. *The survival of the amoebae of* Entamoeba invadens *in culture medium at* 8 *and* 37° C.

	Temperature (° C.)	Time in days	Survival*
Expt. 1	8	22	+
	37	4	+
	37	6	0
Expt. 2	8	35	+
	37	7	+
	37	9	0
Expt. 3	8	7	+
	8	14	0
	37	7	+
	37	14	0

* Determined by microscopic examination or subculture.

The amoebae in the cultures maintained at 24° C. multiplied rapidly, but there was no evidence that they multiplied at 8 or 37° C., nor did they appear to ingest starch at these temperatures. Many abnormal and disintegrating amoebae containing starch were found within a few days of being placed at 37° C. At 8° C., a few vacuolated or disintegrating amoebae were found after 7 days, although the majority were motile and full of starch; after 14 days at this temperature, all the normal amoebae still contained starch, but after 21 days, the proportion of such amoebae had decreased, and those amoebae which had digested their starch, were vacuolated.

(3) *The effect of oxygen on the amoebae*

Amoebae from several cultures were pooled, suspended in Ringer's solution made up according to the formula of Dobell & Laidlaw (1926), and centrifuged down. When these washed amoebae, which were relatively free from bacteria, normal in appearance and motile, were suspended in Ringer's solution maintained at 24° C., it was found that they soon died; 24 hr. after washed amoebae were suspended in Ringer's solution, they became granular or completely disintegrated, while similar changes were sometimes observed 1 hr. after inoculating washed amoebae into Ringer's solution. Washed amoebae were suspended in Ringer's solutions differing in pH, and in modified Ringer's solutions containing different concentrations of

NaCl, or additional salts, but the result was always the same; the amoebae degenerated rapidly, and normal motile amoebae were never found after 24 hr. It was also found that when amoebae were inoculated into Ringer's solution without being washed, they frequently, but not invariably, degenerated within 24 hr.; the amoebae appeared to survive for longer periods when an appreciable amount of starch was simultaneously inoculated, and under such conditions, normal motile amoebae have been found for periods up to at least 14 days, during which time they ingested starch. Similarly, washed amoebae, inoculated with a large amount of starch into Ringer's solution, survived for considerably longer periods than did similar amoebae which were accompanied by only a small amount of starch. Washed amoebae degenerated not only in Ringer's solution, but also in hs and eysaph media; 24 hr. after washed amoebae were inoculated into these media, many abnormal and degenerating amoebae were found, but 6 days later the cultures contained only normal multiplying amoebae.

It seemed probable that the degeneration of amoebae in Ringer's solution and culture media might be due to the presence of free oxygen; when washed amoebae were inoculated into culture media, comparatively few bacteria were present in the inocula, and there was a short period after the inoculation of the cultures before the bacteria had multiplied sufficiently to produce anaerobic conditions, while in Ringer's solution, although the bacteria survived, presumably little or no multiplication occurred. It was found that washed amoebae remained normal and ingested starch when inoculated into Ringer's solution maintained under anaerobic conditions produced either by incubating the tubes of Ringer's solution in a jar containing nitrogen or in which oxygen was absorbed by alkaline pyrogallol, or by absorbing the oxygen in individual tubes with alkaline pyrogallol. Thus when a sample of amoebae was inoculated into anaerobic Ringer's solution, normal active amoebae were still present 21 days later, whereas the amoebae in a similar sample inoculated into aerobic Ringer's solution had started to degenerate within 1 hr., and were all dead within 3 days.

(4) *The sensitivity of the cysts to hydrochloric acid and mercuric chloride*

The survival of the cysts of *E. invadens* in N/20- and N/10-HCl at room temperature was determined in samples of pooled cysts obtained from several cultures of strain A in HSre + S, by a method similar to that described for amoebae. The cysts were very resistant to acid, and survived exposure to N/10-HCl for 6 days; the effect of treatment for longer periods was not determined (Table 6).

The sensitivity of the cysts to 0·002 % $HgCl_2$ at room temperature was determined in samples of cysts which had been freed from amoebae by treatment with N/10-HCl for 1 hr. These amoebae-free cysts were suspended in $HgCl_2$ for varying periods, after which they were thoroughly washed in Ringer's solution, and their viability determined as before, by inoculation into culture medium; cysts suspended in Ringer's solution served as controls. Cysts survived treatment with $HgCl_2$ for 4 hr. and, in one out of 2 experiments, they survived for as long as 24 hr. (Table 6).

Table 6. *The sensitivity of the cysts of* Entamoeba invadens *to hydrochloric acid and mercuric chloride, at room temperature (approximately* 16–20° C.)

	Suspending fluid	Time	Survival*
Expt. 1	Ringer	24 hr.	+
	N/20-HCl	24 hr.	+
Expt. 2	Ringer	24 hr.	+
	N/10-HCl	24 hr.	+
Expt. 3	Ringer	6 days	+
	N/10-HCl	6 days	+
Expt. 4	Ringer	4 hr.	+
	0·002 % $HgCl_2$	4 hr.	+
Expt. 5	Ringer	24 hr.	+
	0·002 % $HgCl_2$	24 hr.	0
Expt. 6	Ringer	24 hr.	+
	0·002 % $HgCl_2$	24 hr.	+

* Determined by subculture.

(5) *The survival of the cysts at* 8, 24 *and* 37° C.

The survival of the cysts of *E. invadens* in Ringer's solution maintained at 8, 24 and 37° C. was determined in samples of amoebae-free cysts, by a method similar to that used for the amoebae. The cysts always survived for at least 14 days at 8° C., whereas at 24° C. they survived for 7 days, but not 11. Viable cysts were never recovered from samples maintained at 37° C. for 7 days or more (Table 7).

Table 7. *The survival of the cysts of* Entamoeba invadens *in Ringer's solution at* 8, 24 *and* 37° C.

	Temperature (° C.)	Time in days	Survival*
Expt. 1	8	11	+
	24	11	0
	37	11	0
Expt. 2	8	7	+
	24	7	+
	37	7	0
Expt. 3	8	14	+
	24	14	0

* Determined by subculture.

(6) *Discussion*

Although these tests were not sufficiently exhaustive to determine the maximum time of survival of amoebae and cysts under all of the different conditions, they do provide data which are of interest when compared with similar data known for *E. histolytica*.

The sensitivity of the amoebae of *E. histolytica* and *E. invadens* to hydrochloric acid appears to be similar. Thus Dobell (1927) found that although the amoebae

of *E. histolytica* were killed within a few minutes of exposure to N/20-HCl at room temperature, they could withstand treatment at their optimum growth temperature, 37° C., for up to 1 hr.; the amoebae of *E. invadens* were usually killed by treatment with N/20-HCl for 10 min. at room temperature, which is near the optimum growth temperature, but on one occasion they survived after treatment for 30 min.

The cysts of the two species appear to differ in their resistance to chemicals. The cysts of *E. histolytica* can survive treatment with N/10-HCl for at least 3 hr. at room temperature, or with N/20-HCl for at least 2 hr. at 37° C. (Dobell, 1927), and with 0·002 % $HgCl_2$ for 45 min. at room temperature (Rao, 1951), although Balamuth & Wieboldt (1951) found that at 25° C. treatment with 0·002 % $HgCl_2$ for 45 min. was lethal to the cysts of this species; it has not, however, been possible to discover data concerning the survival of the cysts after more prolonged exposure to these chemicals. The cysts of *E. invadens* survived exposure to HCl and $HgCl_2$ at room temperature for considerably longer periods than have been demonstrated for *E. histolytica*, and the thicker cyst wall of the former species may render its cysts more resistant to chemicals, as suggested by Dobell & Laidlaw (1926) to explain the differing resistances of the cysts of *E. histolytica*, *E. coli* and *Endolimax nana* to hydrochloric acid.

Although the amoebae of *Entamoeba histolytica* can survive for only 3 days at room temperature (Dobell, 1927)—i.e. at a temperature approximately 20° C. below the optimum temperature for growth—those of *E. invadens* survive for comparatively long periods at 8° C. (also approximately 20° C. below the optimum temperature for growth). Dobell found that amoebae from cultures without starch did not live as long at room temperature as those from cultures containing starch, and he suggested that the state of nutrition of the amoebae might partly determine their resistance to cooling. The majority of the amoebae in cultures of *E. invadens* at 8° C. did not degenerate until they had digested all their starch, and although at this temperature they did not ingest new starch grains, it appeared that they were able to metabolize slowly, and remain alive as long as they contained starch. In contrast, the amoebae of this species degenerated comparatively rapidly at 37° C., while still containing starch, but the heavier growth of bacteria at this temperature probably produced an unfavourable environment more quickly than at 8° C. The present observations do not agree with those of Brumpt (1939), who concluded that the amoebae of *E. invadens* were killed after being kept at 6° C. for only 7 days. The amoebae of *E. invadens* are subjected in the host to much greater changes of temperature than are those of *E. histolytica*, and this may be correlated with their ability to survive for longer periods at temperatures outside the range at which they normally grow.

The survival of the cysts at different temperatures is probably determined largely by the influence of temperature on their metabolic rates. It was observed that at 8° C. the food reserves of the cysts of *E. invadens* were used up more slowly than at 24° C., and the cysts survived for longer periods at the former temperature. Similarly, the cysts of *E. histolytica* remain viable for longer periods at room temperature than at 37° C.

There appear to be two possible explanations of the inability of the amoebae to survive under aerobic conditions; first, that the amoebae cannot metabolize in the presence of free oxygen, or secondly, that oxygen itself, or some metabolite produced when oxygen is present, is toxic to the amoebae. The very rapid degeneration of the amoebae in the presence of oxygen suggests that the latter explanation is the more likely, for if the amoebae were merely unable to metabolize, they would be expected to survive under aerobic conditions for at least several days, just as they survive but do not multiply at 37° C. The harmful effect of oxygen on the amoebae of other species of *Entamoeba* has been noted by several workers (Chang, 1946; Balamuth & Wieboldt, 1951; Neal, 1953), but nothing is known of the mechanism of this toxic effect.

V. THE RELATIONSHIP OF *ENTAMOEBA INVADENS* TO SOME OTHER SPECIES OF *ENTAMOEBA*

It was previously noted that the minor morphological differences observed between *E. invadens* and *E. histolytica* do not justify the distinction of these organisms as independent species. Since the physiological differences between the hosts of *E. invadens* and *E. histolytica* are so great, Geiman & Ratcliffe (1936) considered that the two organisms might be regarded as independent species, but although these workers observed that *E. invadens* grew at temperatures at which the mammalian parasite was unable to grow, no attempt was made to discover the extent of the physiological differences of the two species. The present study permits of a more detailed comparison between *E. invadens* and *E. histolytica*.

It is considered that only two significant differences have been revealed between *E. invadens* and *E. histolytica*, namely a difference in the factors controlling their encystation, and in their temperature requirements. In other respects, the two organisms are strikingly similar; for instance, the amoebae of both are adversely affected by free oxygen, and the growth of either species is enhanced by starch, and sustained, under anaerobic conditions, in similar media in the presence of a mixed bacterial flora or of *Bact. coli* alone. These similarities, which are probably shared by all species of *Entamoeba*, may be correlated with similarities in their natural environments, in which constant anaerobic conditions are maintained, and in which they feed on bacteria, and, in the case of pathogenic amoebae, tissue cells. In addition, the similarities in the response of the two organisms to certain quinaldines (McConnachie, 1954) suggest that they may have similar metabolic requirements.

The factors affecting encystation in cultures of *E. invadens* and *E. histolytica* have already been discussed, and it was suggested that the different behaviour of the two species in this respect might be correlated with certain differences in their natural environments. Although it has been shown that several factors (e.g. growth rate, starch, bacterial flora) may influence encystation in *E. invadens* or *E. histolytica*, it should be emphasized that the precise factor or factors which induce the formation of cysts in either species have not yet been identified, and the difference between the two may not be as great as it appears to be at present.

E. invadens and *E. histolytica* can be differentiated from each other by the temperatures at which they are able to grow, as there is very little overlapping of the temperature ranges of the two species. Dobell & Laidlaw (1926) found that the optimum temperature for the growth of *E. histolytica* is 37–38° C.; no growth occurs at 30° C., but Boeck & Drbohlav (1925) maintained cultures at 32° C. The temperature range of the present strains of *E. invadens* is 16–35° C., with an optimum at 24–30° C. Since *E. invadens* grows at lower temperatures than does *E. histolytica*, it probably has a lower metabolic rate than the latter species, and it has been suggested that this may explain not only the longer life of cultures of *E. invadens*, but also the greater resistance to emetine of this species (McConnachie, 1954). Hitherto, the significance of the different temperature requirements of parasites of warm- and cold-blooded vertebrates does not appear to have been fully appreciated. Thus attempts to infect cold-blooded vertebrates with *E. histolytica*, and warm-blooded vertebrates with *E. invadens*, have both failed, but no mention has been made of the effect of temperature on the parasites under investigation. When, for instance, cysts of *E. invadens* were inoculated into kittens (Ratcliffe & Geiman, 1938), they would have degenerated rapidly, and since the amoebae of this species cannot grow at the temperature of the mammalian body, they are incapable of producing lesions in the mammalian intestine. Similarly, Dobell (1918) observed that the cysts of *E. histolytica* did not excyst when fed to tadpoles, and although he maintained such tadpoles at 32·5° C. for short periods, it is unlikely that excystation could have occurred at this temperature.

Three other species, namely *E. ranarum*, *E. aulastomi* and *E. moshkovskii*, are very similar morphologically to *E. histolytica* and *E. invadens*, and Neal (1953) noted that the typical trophic amoeba and the mature cyst of *E. moshkovskii* cannot be distinguished morphologically from those of the other species. Like *E. histolytica*, however, *E. aulastomi* and *E. moshkovskii* can be distinguished from *E. invadens* by the temperatures at which they are able to grow (Fig. 13). Drbohlav (1925) found that the optimum temperature for the growth of *E. aulastomi* is 20° C., and Bishop (1932) obtained luxuriant growth of this species at 18–22° C.; unlike *E. invadens*, it cannot grow at 30° C. The optimum temperature for *E. moshkovskii* is 24° C., but this organism is distinguished from *E. invadens* by an ability to grow at 37° C., though growth at this temperature is less heavy than at 24° C., and cysts are never formed (Neal, 1953).

The taxonomic status of morphologically identical protozoa has been discussed by Hoare (1943, 1952). In his earlier paper, Hoare considered that groups of organisms which differed only in their ecological preferences should be regarded as 'biological races', which he defined as 'such divisions of a morphological species as are distinguishable by differences in biological characters only', whose taxonomic status should be formally recognized. The biological characters separating certain groups of organisms, however, are undoubtedly stable and hereditarily fixed, and Hoare (1952) later concluded that such biological characters are equivalent to morphological characters as taxonomic criteria; biological races may thus be included in the systematic classification originally based on morphological differences and can be regarded as subspecies.

Whereas there is no morphological character distinguishing *E. invadens*, *E. histolytica*, *E. aulastomi* and *E. moshkovskii*, the biological character (a difference in temperature requirement) separating them is sufficiently stable to justify their recognition as 'hereditarily fixed biological races', or, as Hoare suggests, subspecies. In addition, as noted by Ferris (1928), 'the essence of subspecies is intergradation', and it is well known that subspecies of, for instance, birds and mammals, may show a continuous gradation of morphological characters. The four groups mentioned above form a series whose temperature requirements intergrade from the lower

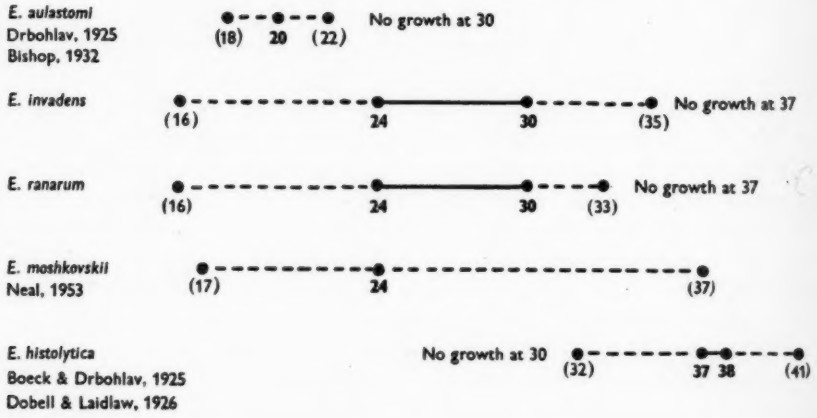

Fig. 13. The temperature requirements of the amoebae of *E. aulastomi*, *E. invadens*, *E. ranarum*, *E. moshkovskii* and *E. histolytica*. Temperatures expressed in ° C. In all species, growth occurs at the temperatures given, but the maximum or minimum temperature at which growth occurs is not known for all (e.g. *E. ranarum* has not been tested between 33 and 37° C.). Figures in parentheses = maximum or minimum temperatures tested for growth. Figures not in parentheses = optimum temperatures.

temperatures at which *E. aulastomi* grows, to the higher temperatures at which *E. histolytica* grows (Fig. 13). It is considered that these organisms should be regarded as subspecies rather than species, and as such should be designated trinominally; *histolytica* has priority over the other specific names, and the free-living *Entamoeba* and the intestinal amoebae from the leech and reptiles should therefore be renamed as subspecies of *E. histolytica*.

It is conceivable that these organisms may show certain differences in their nutritional requirements, but it will be possible to detect such differences only when axenic cultures are available for metabolic studies.

The relationship between *E. invadens* and *E. ranarum* is closer than between any of the other groups previously mentioned, as these two parasites have identical temperature requirements. Sanders (1931) maintained cultures of *E. ranarum* at room temperature, which was not specified, and a strain of *E. ranarum* isolated from a frog by the author has been grown *in vitro* between 16 and 33° C. Cultures of this strain were maintained at 33° C. for 12 weeks by weekly subculture, and showed no diminution in growth during this period, although growth was less

heavy at the higher temperature than at 24° C. or 30° C.; no attempt was made to maintain them between 33 and 37° C., at which latter temperature the amoebae soon died. Geiman & Ratcliffe (1936) obtained some growth of *E. invadens* in the tadpole gut, but cysts of the reptilian *Entamoeba* did not excyst in the tadpole. It remains possible, however, that *E. invadens* and *E. ranarum* are one and the same species, and the results of attempts to infect snakes with *E. ranarum* may throw further light on this question.

VI. SUMMARY

1. Optimum growth of *E. invadens* occurred in HSre + S in the presence of *Bact. coli*, at 24–30° C. The amoebae could not be 'trained' to grow above 35° C.

2. The amoebae ingested normal rat erythrocytes, but not those of chick or frog.

3. Mass encystation occurred only under conditions which favoured the growth of the amoebae, but was not dependent on an acceleration in growth rate; in certain strains, an increased production of cysts was correlated with the depletion of starch in the medium.

4. The amoebae were always killed by treatment with N/20-HCl for 1 hr. at room temperature, although usually they did not survive after treatment for only 10 min. At room temperature, the cysts survived treatment with N/10-HCl for at least 6 days, and with 0·002 % $HgCl_2$ for at least 4 hr.

5. The amoebae of *E. invadens* were adversely affected by aerobic conditions.

6. The amoebae could survive for up to 35 days at 8° C. but for not more than 7 days at 37° C. The cysts survived for at least 14 days at 8° C., for 7, but not 11, days at 24° C., and for less than 7 days at 37° C.

7. It is suggested that, in view of the morphological identity of *E. aulastomi*, *E. invadens*, *E. moshkovskii* and *E. histolytica*, and the fact that the amoebae of these groups form a series growing at increasingly higher temperatures, these four organisms constitute subspecies rather than full species. The taxonomic status of *E. ranarum* is still open to question.

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INTRANUCLEAR CHANGES IN THE POLYHEDROSIS OF *TIPULA PALUDOSA* (MEIG.)

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(With Plates VIII–XI)

I. INTRODUCTORY

The technique of cutting ultra-thin sections for electron microscopy has made it possible to follow the behaviour of certain viruses in the cell, and this is particularly true of the nuclear virus diseases of insects.

In the present paper a description is given of the remarkable behaviour in the nucleus of the polyhedral virus which causes a blood disease in the larva of the fly *Tipula paludosa*.

This disease was first discovered and briefly described by Rennie (1923), although he did not realize that the virus affected the blood cells. It was rediscovered, and some preliminary work carried out, by Smith & Xeros (1954*a*) and Xeros & Smith (1955).

The affected larvae can be recognized by their unusual pallor which increases as the disease progresses, until in the last stages the larva may be almost white. For a long time, however, the diseased larva continues to live an apparently normal life.

II. MATERIAL AND METHODS

Young larvae, about one-third grown, were used, since it was more difficult to infect older larvae. Infection could be brought about fairly easily by dipping the head end into a semi-purified suspension of polyhedra. The larvae were reared in Petri dishes on grass roots from which all soil had been removed by washing; this method was suggested by Mr T. Parsons. Removal of the soil greatly facilitates section cutting for light microscopy. At first filter-paper was used to line the Petri dishes to keep up the humidity, but this was discarded since the larvae ate the paper and thus presented a white appearance which closely resembled the polyhedral disease. The larvae lived well under these conditions and the disease developed in about 14 days during the spring and summer, but might be delayed for a month or more during the winter.

For examination on the optical microscope the larvae were fixed in osmic acid or Carnoy's fluid. Blood smears were stained with Giemsa's solution.

For electron microscopy all material was fixed in 2% osmic acid buffered at pH 7 with phosphate buffer. The larvae were first injected with osmic acid and later cut up and left to fix for 2–3 hr.

For sectioning the blood cells, these were centrifuged in osmic acid and then taken up to the embedding stage, centrifuging after each transfer. To obtain sec-

tions of the virus particles themselves freed from cellular material, these were spun down, also in osmic acid, at a speed of 12,000 r.p.m. for 40 min. The subsequent pellet was then treated similarly to the other material. Each object was embedded in plastic consisting of 8 parts butyl methacrylate to 2 parts of methyl methacrylate. Sections were cut on a Cook and Perkins microtome fitted with a glass knife, and photographed on a Metropolitan Vickers E.M. 3 electron microscope using 75 kV. They were not shadowed with metal, since the plastic was not removed. Resolution difficulties are apt to arise in photographing ultra-thin sections, especially if the sections are thinner than the formvar film supporting them. It was necessary therefore to take a focal series in most cases. Carbon films in place of formvar were also used on occasion. Acknowledgement is due to Miss S. Vernon-Smith who took the photographs.

III. BEHAVIOUR OF THE VIRUS IN THE NUCLEUS

Earlier workers had noticed that in the polyhedral diseases of lepidopterous larvae such as *Lymantria dispar* and *L. monacha*, there develops a pathological body consisting of agglomerated chromatin in the diseased nuclei. Sections were made of this body and photographed on the electron microscope for the first time by Smith, Wyckoff & Xeros (1953). Owing to its appearance under the electron microscope it was later referred to as the 'nuclear net'. This chromatic mass, or net, is characteristic of the nuclear polyhedrosis of lepidopterous larvae and there is a good deal of evidence that it is the site of development and multiplication of the virus (Smith & Xeros, 1953, 1954*b*; Hughes, 1953; Bird & Whalen, 1954).

In a recent paper (Xeros & Smith, 1955) electron micrographs of sections of diseased nuclei, from larvae of *Tipula paludosa* infected with polyhedrosis, are shown. These sections were cut on a modified rotary microtome, and the plastic was removed with amyl acetate. The micrographs show a central body, frequently horse-shoe shaped, with a less dense pericentral body; outside these is a false ring zone with virus rods embedded in it.

With the ultra-thin sections now obtainable with the latest type of microtome and with the methacrylate left *in situ*, the appearance of the nucleus is slightly different. There is a rather dense area in the centre of the nucleus which appears to be a kind of condensation of the chromatin and here particles which are much denser in character become differentiated and these emerge as virus rods (Pl. VIII, figs. 1, 2); they may be single, or in bundles and packets of six or more.

No pericentral body has been observed and this may possibly be an artifact caused during the removal of the methacrylate. In every one of a long series of sections through diseased blood cells the virus rods were found to be concentrated in the centre of the nucleus, though single rods occur scattered throughout the whole nucleus. No virus rods have ever been observed on the outer side of the nuclear membrane.

It has been shown previously (Smith & Xeros, 1954*a*) that the rather remarkable 'polyhedra' of this disease, which actually are crescent shaped, are situated with one edge on the nuclear membrane and the other in the cytoplasm of the blood cell.

By examining a large number of sections of blood cells in various stages of the disease the changes taking place in the nucleus, before the formation of the 'polyhedra', have been worked out. These changes are remarkable and extremely interesting.

As the disease progresses, a large apparently thin-walled vesicle forms round individual virus rods (Pl. IX, fig. 4; Pl. X, fig. 5). These vesicles are much less dense than the surrounding medium, and the virus rod in each vesicle seems thinner and more sharply defined than the rods remaining outside. The appearance of the vesicle, and the fact that the rod is suspended away from the sides suggest that there may be fluid, possibly a solution of protein, inside the vesicle. A large number of the vesicles collect together on the inside edge of the nucleus where a membrane apparently forms round them. At all events something seems to hold the vesicles together. The edge of the mass nearest to the nuclear membrane then becomes denser and the vesicles at this point are apparently compressed and much smaller. As the crystallization proceeds the vesicles become greatly reduced in size, possibly because of the osmotic effect of the medium surrounding them, till each vesicle, empty now of fluid, and closely pressed round the virus rod, eventually forms the capsule in which each rod in the polyhedral crystal is enclosed (Pl. X, fig. 7). It may be that the fluid forced out of the vesicle is used in the crystal formation. All these changes take place *inside* the nucleus (Pl. X, fig. 6), but, as already stated, the position of the completed crystal is *outside* the nucleus with one edge on the nuclear membrane. Apparently, then, as the crystals form they are forced out of the nucleus till they reach that position. In Pl. XI, fig. 8, the completed crystals can be seen outside the nucleus. It has been assumed in the case of the formation of the polyhedra in polyhedroses of lepidopterous larvae that the crystals form in a haphazard manner and any virus particles which may be in the vicinity are occluded during the crystal formation. In the disease under discussion, however, the situation is very different. Here the virus particles obviously collect together before the formation of the polyhedra, each one in its enveloping vesicle, and only when there is a mass of particles does the crystal form round it.

In Pl. XI, figs. 11 and 12, are shown sections through the edge of a polyhedral crystal, and the capsules, some with the top or side cut away, can be clearly seen.

A number of sections have been cut through the polyhedral crystals to ascertain whether there is any regular arrangement of the virus rods inside. This might be the case in view of the fact that the crystals show negative birefringence. However, except in one or two cases where there does appear some regularity, the arrangement of the particles seems to be haphazard (Pl. XI, figs. 9, 10).

IV. SUBMICROSCOPIC STRUCTURE OF THE VIRUS PARTICLES

By the use of a combination of modern techniques, such as the high resolution obtainable with some electron microscopes, ultra-thin sections and the use of carbon films on the grids, it is now possible to gain some knowledge of the internal structure of the virus particle itself. One fact is particularly striking and that is

the tendency of the particles to be contained within double membranes. This tendency is not confined to insect viruses, it has been shown by Morgan Ellison, Rose and Moore (1954*a*) that the virus of *Herpes simplex* appears to develop in the cell nucleus with a single membrane and when released into the cytoplasm to acquire a second outer membrane. The same workers (1954*b*) have found a similar stage of affairs with vaccinia and fowl pox viruses.

Sections through the centres of diseased nuclei of *T. paludosa* reveal the virus particles scattered throughout the chromatin. In Pl. VIII, figs. 1 and 2, isolated virus rods can be seen, together with packets of six or more. Although no membrane is visible round these rods, there is presumably an intimate membrane which holds together the substance of the virus rod itself. In Pl. VIII, fig. 3, which shows sections of virus rods that have been separated by centrifugation from cellular material, an outer envelope or membrane is very evident. The fact that in sections this membrane stands out some distance from the substance of the rod suggests that there may be in addition an inner intimate membrane closely approximated to the rod itself. If that is so the second membrane may be developed later after formation of the rod. In the sections of the vesicles the virus rod within appears to be without a second membrane (Pl. IX, fig. 4), although the rods lying outside the vesicles are definitely enclosed within a membrane.

If the assumption is correct that it is the vesicle which, by being compressed round the virus rod, forms the capsule, then one would expect virus rods outside the polyhedral crystal to have a membrane surrounding them but no capsule, and this appears to be the case (Pl. VIII, fig. 3). Capsules surrounding the virus rods are characteristic both of the polyhedral and granulosis diseases of lepidopterous larvae. In the silkworm *Bombyx mori* it has been shown that the virus rods in the ring zone of the nucleus are without capsules, but these are apparently acquired later inside the polyhedral crystal (Smith & Xeros, 1954*b*). There is as yet no evidence, however, of a process in the silkworm analogous to that described here for the larva of *Tipula paludosa*.

V. SUMMARY

By means of ultra-thin sections for electron microscopy it has been possible to observe intra-nuclear changes in the polyhedrosis of larvae of *Tipula paludosa*. The virus rods arise in the centres of the greatly enlarged nuclei of blood cells. Thin-walled vesicles, apparently filled with fluid, form round individual virus rods and collect in masses on the inner wall of the nuclear membrane. Crystallization then begins, starting at the edge against the nuclear membrane. The vesicles are gradually compressed round the rods, eventually forming a capsule. Sections through free virus particles show them to be enclosed in a membrane, a second inner membrane round the substance of the rod may also be present.

There appears to be no capsule round the free virus particles such as encloses the virus particles inside the polyhedral crystal.

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EXPLANATION OF PLATES VIII—XI

(Electron micrographs by S. Vernon-Smith)

PLATE VIII

Fig. 1. Part of a section through the centre of a diseased nucleus; note the virus rods developing, sometimes singly, sometimes in rows of six or more. × 50,000.

Fig. 2. A similar section to that in fig. 1, but photographed at a higher magnification. × 67,000.

Fig. 3. Sections through individual virus particles separated from the cells; note the enveloping membrane and the wide space between it and the substance of the virus which appears to be made up of granules. × 120,000.

PLATE IX

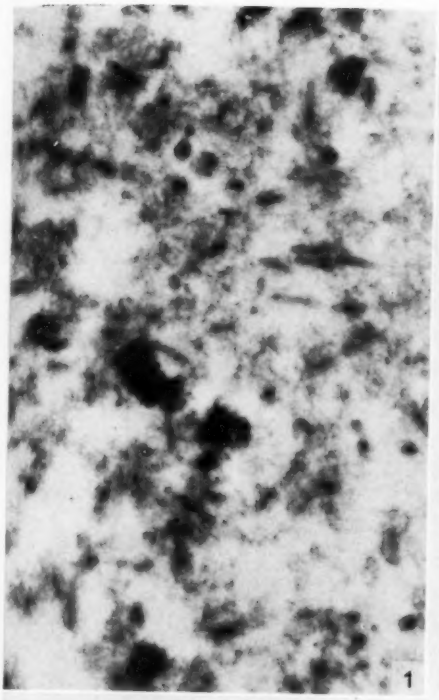
Fig. 4. Section through a complete blood cell, prior to the formation of polyhedra; note five masses of vesicles situated inside the enlarged nucleus; in each vesicle is one virus rod which appears thinner than those lying ouside. × 16,600.

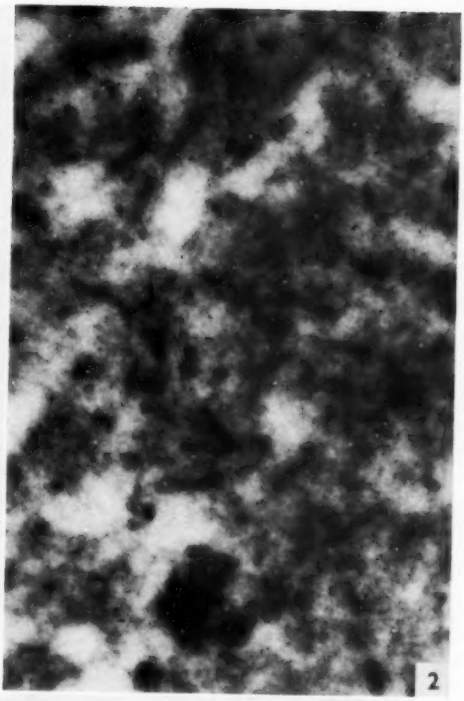
PLATE X

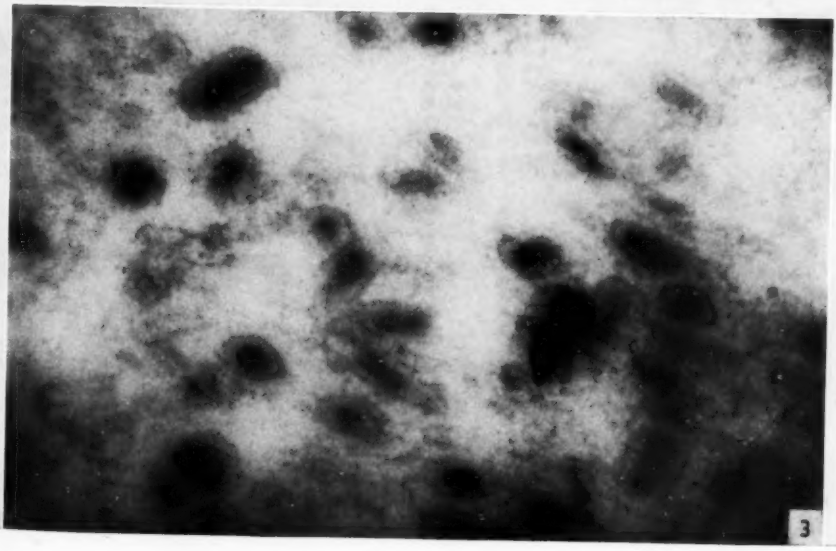
Fig. 5. Section through one half of a diseased blood cell showing three vesicular masses; note the denseness of the area against the nuclear membrane where crystallization is apparently starting. × 22,000.

Fig. 6. Segment of a similar cell, this shows very well the apparent compression of the vesicles near the nuclear membrane. × 16,600.

Fig. 7. Here crystallization is nearly complete; note denseness of the surrounding matrix, which appears to have a definite wall, and the compression of the vesicles. The crystal is now half-way over the nuclear membrane. × 22,000.







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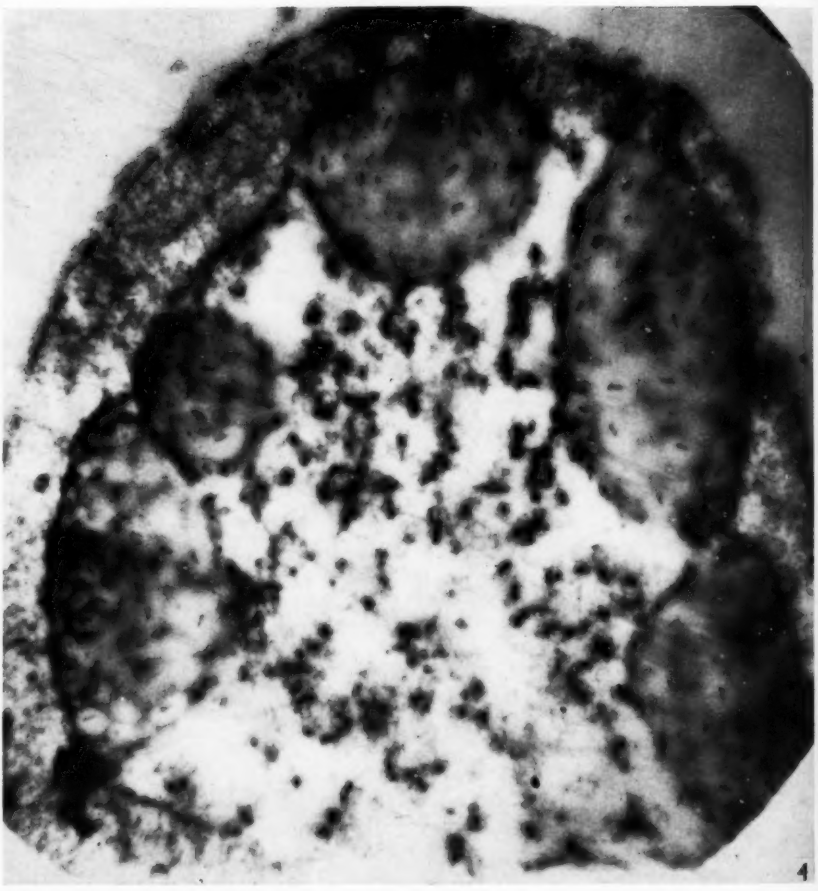
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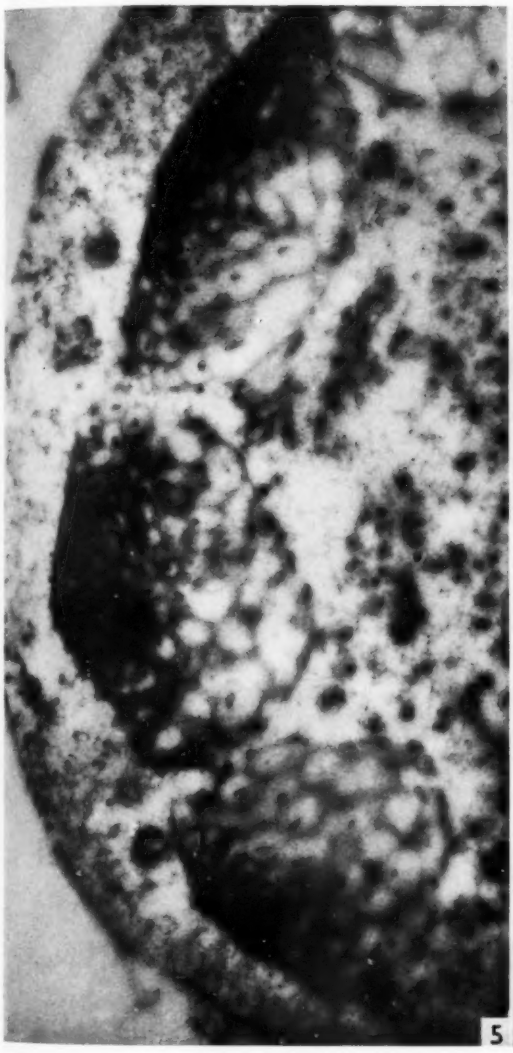
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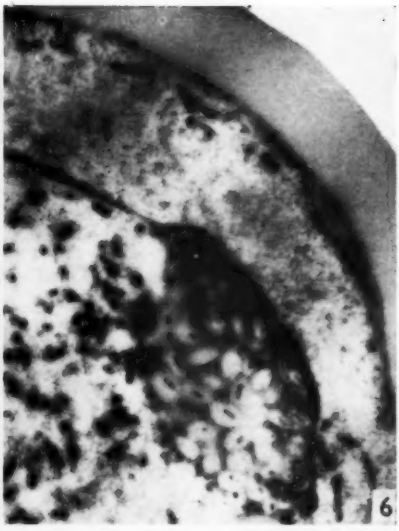
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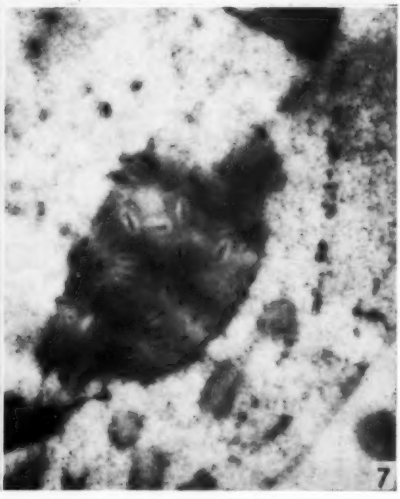
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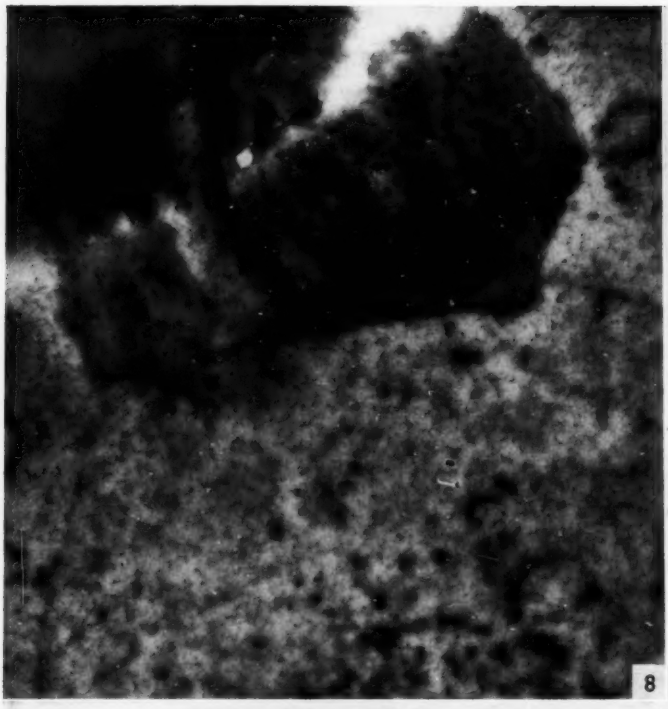
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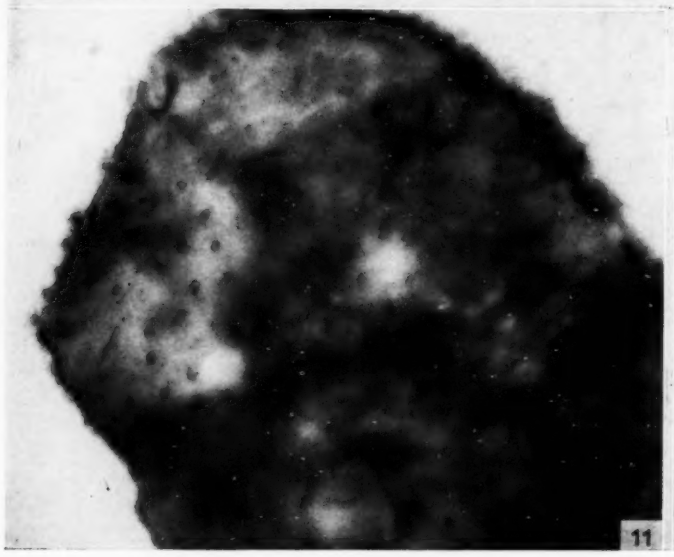
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PLATE XI

Fig. 8. Section through part of a blood cell at a late stage of the disease; the polyhedra have now taken up their position on the nuclear membrane. Note the virus rods in the crystal and how they appear to have been mostly cut transversely. × 25,000.

Figs. 9 and 10. Sections through two polyhedra showing the apparently haphazard arrangement of the virus particles within. × 16,800.

Fig. 11. Part of a section through a polyhedral crystal; note at top left the sectioned virus rods embedded in their capsules. × 50,000.

Fig. 12. Thin section through one edge of a polyhedral crystal; note the capsules, some of which appear to be empty, cut at various angles. × 33,000.

INDEX OF AUTHORS

ARTHUR, DON R. *Ixodes hoogstraali*, a new species of tick from Yemen. (With 11 Figures in the Text), 128

ARTHUR, DON R. New species of ticks (*Ixodes*) associated with small mammals. (With 31 Figures in the Text), 131

BAILEY, L. The infection of the ventriculus of the adult honeybee by *Nosema apis* (Zander). (With Plate IV and 3 Figures in the Text), 86

BEER, JAMES R. *See* COOK, EDWIN F.

BISHOP, ANN. Problems concerned with gametogenesis in Haemosporidiidea, with particular reference to the genus *Plasmodium*, 163

COOK, EDWIN F. and BEER, JAMES R. The louse populations of some cricetid rodents. (With 4 Figures in the Text), 409

CORBETT, M. PAULINE. Occurrence of two species of *Crepidostomum* in brown trout (*Salmo trutta* L.) from north-east Ireland, with special reference to *Crepidostomum metoecus* Braun 1900, 186

CROFTON, H. D. Nematode parasite populations in sheep on lowland farms. II. Worm egg counts in lambs. (With 9 Figures in the Text), 99

DASGUPTA, B. *See* RAY, H. N.

DAVIES, A. MICHAEL. *See* ELIAKIM, M.

DE OLIVEIRA, M. XAVIER and MEYER, H. *Plasmodium gallinaceum* in tissue culture. Observations after one year of cultivation. (With Plate I), 1

ELIAKIM, M. and DAVIES, A. MICHAEL. The complement-fixation test in Bilharziasis. II. Preparation and preservation of antigens from *Schistosoma mansoni* worms extracted in Coca's solution, 189

EVANS, G. OWEN. A review of the Laelaptid paraphages of the Myriapoda with descriptions of three new species (Acarina: Laelaptidae). (With 30 Figures in the Text), 352

FRANKLAND, HELGA M. T. The life history and bionomics of *Diclidophora denticulata* (Trematoda: Monogenea). (With 21 Figures in the Text), 313

GOODCHILD, A. J. P. The bacteria associated with *Triatoma infestans* and some other species of Reduviidae, 441

HAWKING, FRANK. *See* WEBBER, WINFRITH A. F.

HAWKING, FRANK and WEBBER, WINFRITH A. F. *Dirofilaria aethiops* Webber, 1955, a filarial parasite of monkeys: II. Maintenance in the laboratory. (With 1 Figure in the Text), 378

INGLIS, WILLIAM G. On the family Parasubuluridae van den Berghe & Vuylsteke, 1938, and the subfamily Numidicinae López-Neyra, 1945 (Nematoda). (With 9 Figures in the Text), 431

KETTLE, D. S. and UTSI, M. N. P. *Hypoderma diana* (Diptera, Oestridae) and *Lipoptena cervi* (Diptera, Hippoboscidae) as parasites of reindeer (*Rangifer tarandus*) in Scotland with notes on the second-stage larva of *Hypoderma diana*, 116

LAVOIPIERRE, M. M. J. On two new species and new records of Acarina mainly from the Ethiopian region. (With 9 Figures in the Text), 121

MCCONNACHIE, ELSPETH W. Studies on *Entamoeba invadens* Rodhain, 1934, *in vitro*, and its relationship to some other species of *Entamoeba*. (With 13 Figures in the Text), 452

MAHON, JUNE. Contributions to the genus *Paradilepis* Hsü, 1935. (With 16 Figures in the Text), 63

MELLANBY, HELEN. The identification and estimation of acetylcholine in three parasitic nematodes (*Ascaris lumbricoides, Litomosoides carinii*, and the microfilariae of *Dirofilaria repens*). (With 6 Figures in the Text), 287

MENDONÇA, I. DE ANDRADE. *See* MEYER, H.

MEYER, H. *See* DE OLIVEIRA, M. XAVIER

MEYER, H. and MENDONÇA, I. DE ANDRADE. Electron microscopic observations of Toxoplasma 'Nicolle et Manceaux' grown in tissue cultures (first note). (With Plate VII), 449

MUKERJI, D. and SEN-SARMA, P. Anatomy and affinity of the elephant louse *Haematomyzus elephantis* Piaget (Insecta: Rhyncophthiraptera). (With 40 Figures in the Text), 5

NEAL, R. A. and VINCENT, PATRICIA. Strain variation in *Entamoeba histolytica*. I. Correlation of invasiveness in rats with the clinical history and treatment of the experimental infections, 152

QADRI, SYED SHAMSUDDIN. The morphology of *Trypanosoma striati* n.sp., from an Indian fresh-water fish. (With 12 Figures in the Text), 79

RADFORD, CHARLES D. Some new and little-known mites of the genus *Myocoptes* Claparède (Acarina: Listrophoridae). (With 16 Figures in the Text), 275.

RAY, H. N. and DASGUPTA, B. Observations on the structure and the nature of '*NR* bodies' found in the testes of bed-bug. (With Plate VI), 421

REES, GWENDOLEN. The adult and *Diplostomulum* stage (*Diplostomulum phoxini* (Faust)) of *Diplostomum pelmatoides* Dubois and an experimental demonstration of part of the life cycle. (With Plate V and 10 Figures in the Text), 295

SARWAR, M. M. On a new lungworm, *Pneumostrongylus ovis* Sarwar, 1953, and some remarks on *Varestrongylus* species from sheep and goats in Pakistan. (With 3 Figures in the Text), 426

SEN-SARMA, P. *See* MUKERJI, D.

SMITH, KENNETH M. Intranuclear changes in the polyhedrosis of *Tipula paludosa* (Meig.). (With Plates VIII–XI), 482

SPRENT, J. F. A. On the invasion of the central nervous system by nematodes. I. The incidence and pathological significance of nematodes in the central nervous system, 31

SPRENT, J. F. A. On the invasion of the central nervous system by nematodes. II. Invasion of the nervous system in ascariasis. (With Plates II and III and 5 Figures in the Text), 41

STEWARD, J. S. Anthelmintic studies: I. A controlled critical entero-nemacidal test, 232

STEWARD, J. S. Anthelmintic studies: II. A double entero-nemacidal anthelmintic test covering a wide range of activities. (With 1 Figure in the Text), 242

STEWARD, J. S. Anthelmintic studies: III. A taeniacidal testing technique, 255

STEWARD, J. S. Anthelmintic studies: IV. The loss of efficiency by division of the dose, 266

THEODOR, O. On the genus *Eucampsipoda* Kol. and *Dipseliopoda* n.g. (Nycteribiidae, Diptera). (With 53 Figures in the Text), 195

THURSTON, JUNE P. Observations on the course of *Eperythrozoon coccoides* infections in mice, and the sensitivity of the parasite to external agents. (With 5 Figures in the Text), 141

TILL, W. *See* ZUMPT, F.

UTSI, M. N. P. *See* KETTLE, D. S.

VINCENT, PATRICIA. *See* NEAL, R. A.

WALKER, JANE B. *Rhipicephalus pulchellus* Gerstäcker 1873: a description of the larva and nymph with notes on the adults and on its biology. (With 7 Figures in the Text), 95

WEBBER, WINFRITH A. F. *Dirofilaria aethiops* Webber, 1955, a filarial parasite of monkeys: I. The morphology of the adult worms and microfilariae. (With 12 Figures in the Text), 369

WEBBER, WINFRITH A. F. *Dirofilaria aethiops* Webber, 1955, a filarial parasite of monkeys: III. The larval development in mosquitoes. (With 16 Figures in the Text), 388

WEBBER, WINFRITH A. F. and HAWKING, FRANK. The filarial worms *Dipetalonema digitatum* and *D. gracile* in monkeys. (With 7 Figures in the Text), 401

WEBBER, WINFRITH A. F. *See* HAWKING, FRANK

WILLIAMS, HOLMAN E. Studies on the bovine filariid *Setaria cervi* (Rudolphi, 1819). (With 12 Figures in the Text), 56

ZUMPT, F. and TILL, W. The mange-causing mites of the genus *Psorergates* (Acarina: Myobiidae) with description of a new species from a South African monkey. (With 7 Figures in the Text), 269

INDEX OF SUBJECTS

Acarina, on two new species and new records of, mainly from the Ethiopian region, 121
Acetylcholine, identification and estimation of, in three parasitic nematodes (*Ascaris lumbricoides*, *Litomosoides carinii*, and the microfilariae of *Dirofilaria repens*), 287
Anatomy and affinity of the elephant louse *Haematomyzus elephantis* Piaget (Insecta: Rhyncophthiraptera), 5
Anthelmintic studies: I. A controlled critical entero-nemacidal test, 232
Anthelmintic studies: II. A double entero-nemacidal anthelmintic test covering a wide range of activities, 242
Anthelmintic studies: III. A taeniacidal testing technique, 255
Anthelmintic studies: IV. The loss of efficiency by division of the dose, 266

Bacteria associated with *Triatoma infestans* and some other species of Reduviidae, 441
Bed-bug, '*NR* bodies' found in testes of, 421
Bilharziasis, the complement-fixation test in, II. Preparation and preservation of antigens from *Schistosoma mansoni* worms extracted in Coca's solution, 189

Crepidostomum, occurrence of two species of, in brown trout (*Salmo trutta* L.) from north-east Ireland, with special reference to *Crepidostomum metoecus* Braun 1900, 186

Diclidophora denticulata (Trematoda: Monogenea), life history and bionomics of, 313
Dipetalonema digitatum and *D. gracile*, the filarial worms, in monkeys, 401
Diplostomum pelmatoides Dubois, the adult and *Diplostomulum* stage (*Diplostomulum phoxini* (Faust)) of, and an experimental demonstration of part of the life cycle, 295
Dirofilaria aethiops Webber, 1955, a filarial parasite of monkeys. I. The morphology of the adult worms and microfilariae, 369
Dirofilaria aethiops Webber, 1955, a filarial parasite of monkeys. II. Maintenance in the laboratory, 378
Dirofilaria aethiops Webber, 1955, a filarial parasite of monkeys. III. The larval development in mosquitoes, 388

Electron microscopic observations of Toxoplasma 'Nicolle et Manceaux' grown in tissue cultures (first note), 449
Elephant louse *Haematomyzus elephantis*, anatomy and affinity, 5
Entamoeba histolytica, strain variation in. I. Correlation of invasiveness in rats with the clinical history and treatment of the experimental infections, 152
Entamoeba invadens Rodhain, 1934, *in vitro*, studies on, and its relationship to some other species of *Entamoeba*, 452
Eperythozoon coccoides infections in mice, observations on the course of, and the sensitivity of the parasite to external agents, 141
Eucampsipoda Kol., on the genus, and *Dipselipoda* n.g. (Nycteribiidae, Diptera), 195

Filarial worms, *Dipetalonema digitatum* and *D. gracile*, in monkeys, 401
Fish, *Trypanosoma striati* n.sp. from, 79

Haematomyzus elephantis Piaget (Insecta: Rhyncophthiraptera) anatomy and affinity of the elephant louse, 5
Haemosporidiidea, problems concerned with gametogenesis in, with particular reference to the genus *Plasmodium*, 163
Honeybee, infection by *Nosema apis*, 86
Hypoderma diana (Diptera, Oestridae) and *Lipoptena cervi* (Diptera, Hippoboscidae) as parasites of reindeer (*Rangifer tarandus*) in Scotland with notes on the second-stage larva of *Hypoderma diana*, 116

Ixodes hoogstraali, a new species of tick from Yemen, 128

Laelaptid paraphages of the Myriapoda with descriptions of three new species (Acarina: Laelaptidae), a review of the, 352
Life history and bionomics of *Diclidophora denticulata* (Trematoda: Monogenea), 313
Louse populations of some cricetid rodents, 409
Lungworm, on a new, *Pneumostrongylus ovis*, Sarwar, 1953, and some remarks on *Varestrongylus* species from sheep and goats in Pakistan, 426

Mange-causing mites of the genus *Psorergates* (Acarina: Myobiidae) with description of a new species from a South African monkey, 269
Mice, infection by *Eperythrozoon coccoides*, 141
Mites of the genus *Psorergates* causing mange in monkey, 269
Mites, some new and little-known, of the genus *Myocoptes* Claparède (Acarina: Listrophoridae), 275
Monkeys, filarial worms in, 401
Morphology of *Trypanosoma striati* n.sp., from an Indian fresh-water fish, 79
Myocoptes Claparède (Acarina: Listrophoridae), some new and little-known mites of the genus, 275

Nematode parasite populations in sheep on lowland farms. II. Worm egg counts in lambs, 99
Nematodes, on the invasion of the central nervous system by. I. The incidence and pathological significance of nematodes in the central nervous system, 31
Nematodes, on the invasion of the central nervous system by. II. Invasion of the central nervous system in ascariasis, 41
Nematodes (*Ascaris lumbricoides*, *Litomosoides carinii*, and the microfilariae of *Dirofilaria repens*) identification and estimation of acetylcholine in three parasitic, 287
Nosema apis, the infection of the ventriculus of the adult honeybee by, 86
'*NR* bodies' found in the testes of bed-bug, observations on the structure and the nature of, 421

Paradilepis, Hsü, 1935, contributions to the genus, 63
Parasubuluridae van den Berghe & Vuylsteke, 1938, on the family, and the subfamily Numidicinae López-Neyra, 1945 (Nematoda), 431
Plasmodium gallinaceum in tissue culture. Observations after one year of cultivation, 1
Pneumostrongylus ovis, Sarwar, 1953, on a new lungworm, and some remarks on *Varestrongylus* species from sheep and goats in Pakistan, 426
Polyhedrosis of *Tipula paludosa*, intranuclear changes in, 482
Psorergates (Acarina: Myobiidae), the mange-causing mites of the genus, with description of a new species from a South African monkey, 269

Review of the Laelaptid paraphages of the Myriapoda with descriptions of three new species (Acarina: Laelaptidae), 352
Rhipicephalus pulchellus Gerstäcker, 1873; a description of the larva and nymph with notes on the adults and on its biology, 95

Setaria cervi (Rudolphi, 1819), studies on the bovine filariid, 56

Ticks (*Ixodes*) new species of, associated with small mammals, 131
Tipula paludosa (Meig.), intranuclear changes in the polyhedrosis of, 482
Triatoma infestans, the bacteria associated with, and some other species of Reduviidae, 441
Trypanosoma striati n.sp., the morphology of, from an Indian fresh-water fish, 79
Toxoplasma 'Nicolle et Manceaux', grown in tissue culture (first note), electron microscopic observations of, 449